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# A New Method for the Direct Isolation of Glycine from Protein Hydrolysates

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A number of procedures have been proposed for the isolation of glycine from protein hydrolysates. The ester method originally described by Fischer (1901) is laborious and involves great losses, owing largely to incomplete precipitation of glycine ester hydrochloride and the volatility of the free ester. The precipitation of glycine as its barium carbamate (Kingston & Schryver, 1924) is time-consuming. Nitranilic acid, introduced by Town (1936) as a specific precipitant for glycine, has been shown by subsequent workers (Stein & Miller, 1938; Macara & Plimmer, 1940; Block, 1940) to precipitate histidine along with glycine. The difficulties inherent in the earlier methods may be partially overcome by the direct precipitation of glycine as a complex with potassium trioxalatochromiate (Bergmann & Fox, 1935). This method, however, suffers from the disadvantage of introducing potassium ions, which are difficult to remove, into the solution.

The purpose of this paper is to describe a simple procedure for the direct isolation of glycine from protein hydrolysates. The present work originated from a study of the direct isolation of lysine picrate from gelatin hydrolysates (Robson & Selim, 1952). While isolating this picrate, it was found that when calcium hydroxide was used instead of sodium hydroxide to attain the appropriate pH a crystalline yellow compound was instantaneously formed. Preliminary examination of the product indicated that it was a complex compound of calcium picrate with one or more of the neutral amino acids. This property of calcium picrate has already been reported (Robson & Selim, 1953).

In order to throw further light on this problem, it was decided to investigate the behaviour of calcium picrate towards the α-amino acids which are usually present in a protein hydrolysate. It was found that this picrate forms a highly insoluble complex salt with glycine. A number of other monoamino acids were found to form similar complex derivatives, although the solubilities of the latter in water are not so low as that of the calcium picrate–glycine complex. While further study of the conditions under which calcium picrate can be employed for the analysis of certain neutral amino acids is still in progress, a search has been made for other metal picrates capable of specifically precipitating glycine from protein hydrolysates.

Copper picrate, which is readily soluble in water, has been found to fulfil the above requirement. Under the conditions employed, glycine alone, of all the amino acids normally occurring in a protein hydrolysate, forms a sparingly soluble complex salt with copper picrate. Analysis of this complex proves it to be composed of 2 moles of glycine combined with 1 mole of copper picrate, its formula being $(C_2H_5O_2N)_2,Cu(C_6H_2O_7N_3)_2,6H_2O$. It is readily obtained from aqueous solutions containing the two components in the ratio of 2 moles of glycine to 1 mole of copper picrate, but the same compound can also be isolated from solutions having the precipitant and glycine in different molecular proportions. Glycine–copper picrate complex crystallizes promptly from hot water in yellowish green plates. It is soluble in water to the extent of 0·9 g./100 ml. at 10°, but in the presence of a slight excess of copper picrate the solubility diminishes to 0·25 g./100 ml. Experiments with aqueous solutions containing 2 g. of glycine have indicated that under optimum conditions the amino acid can be recovered to the extent of 98·5% by treatment with copper picrate.

These facts suggest a new procedure for the direct precipitation of glycine from protein hydrolysates. In order to demonstrate the practical applicability of the method, two representative proteins were analysed, one (gelatin) rich in glycine and the other (egg albumin) relatively poor in this amino acid. In each case, glycine–copper picrate complex was obtained in good yield and purity.

With gelatin, the method consists mainly in treating a warm concentrated hydrolysate, previously freed from the sulphuric acid used for hydrolysis, with twice as much copper picrate as that required to combine with the glycine present. When all the precipitant has dissolved and the mixture has cooled to room temperature, yellowish green crystals of the glycine complex derivative begin to separate. After a few hours the heavy crystalline precipitate is collected and washed with ice-cold water. Recrystallization of the product from hot water gives pure glycine–copper picrate.

Assuming that the glycine content of moisture- and ash-free purified gelatin, calculated to 16·0 g. of nitrogen, is between 22·2 and 22·8 % (Block & Bolling, 1951), the yield of the final product corresponds to 92–94 % of the total amino acid present. Separation of the free amino acid is easily effected by decomposition of the complex salt with hot sulphuric acid, extraction of picric acid with hot benzene and removal of copper as sulphide. From the sulphuric acid-free solution pure glycine has been obtained in yields equivalent to 90 % of the theory.

With proteins having a smaller glycine content, such as egg albumin, the technique described above is varied slightly. When copper picrate is added to a concentrated egg-albumin hydrolysate in the proportion of one mole to each mole of glycine present no precipitation takes place. This is due to competition between glycine and the basic amino acids for the precipitant. To overcome this difficulty, several times as much copper picrate as that theoretically required for the formation of the glycine complex are dissolved in the hydrolysate. Furthermore, it has been found that the presence of large amounts of ammonia interferes with the precipitation of the complex salt through the formation of ammonium picrate. The isolation of the glycine complex derivative should, therefore, be preceded by the removal of ammonia. From egg-albumin hydrolysates, pure glycine–copper picrate complex has been isolated in amounts corresponding to approximately 90 % of the total amino acid present in the protein. This figure has been calculated on the assumption that the glycine content of purified egg albumin, corrected for moisture and ash, and calculated to 16 g. of nitrogen, is between 3·3 and 3·6 % (Block & Bolling, 1951).

The recrystallization of the glycine–copper picrate complex should be carried out from relatively large volumes of water at temperatures not exceeding 90°. When the complex salt is dissolved in a small volume of boiling water it undergoes partial decomposition, with the formation of a basic form of glycine–copper picrate. Treatment of the complex salt with boiling ethanol results in its complete conversion into a mixture of the basic form of glycine–copper picrate, glycine picrate and picric acid. It has been shown that the presence of a little water is an important factor in this decomposition, since the anhydrous complex is not affected by boiling with ethanol. The reaction may be represented by the equation

$$2(C_2H_5O_2N)_2,Cu(C_6H_2O_7N_3)_2,6H_2O = (C_2H_5O_2N)_2,Cu(C_6H_2O_7N_3)_2,CuO + (C_2H_5O_2N)_2,C_6H_3O_7N_3 + C_6H_3O_7N_3 + 5H_2O.$$

A study of the properties of a number of other heavy-metal picrates has shown that some of them can be successfully employed as specific precipitants for certain monoamino acids. Further results will be reported later.

## EXPERIMENTAL

### *Materials*

Gelatin (E. Merck, Darmstadt) (N, 16·5 % of moisture-free protein; moisture, 14·6 %; ash, 2·08 %).

Egg albumin (British Drug Houses Ltd.) (N, 14·08 % of moisture-free protein; moisture, 10·9 %; ash, 4·95 %).

Copper picrate ($Cu(C_6H_2O_7N_3)_2,5H_2O$) was prepared by the following procedure: to a suspension of commercial picric acid in hot water, an excess of pure copper carbonate was added in small portions with stirring. The mixture was then boiled for a few minutes and filtered while hot. After several hours in the refrigerator, the resulting green crystalline precipitate of copper picrate was filtered off, washed with a little ice-cold water and then air-dried.

### *Isolation of glycine–copper picrate complex from gelatin hydrolysates*

Gelatin (200 g.) was added to 1200 ml. of 8 N-$H_2SO_4$ and the protein brought completely into solution by heating on a steam bath. When the protein had dissolved the mixture was refluxed for 30 hr. The hydrolysate was diluted with 2 vol. of distilled water and the insoluble humin removed by filtration. The acid mixture was neutralized to litmus by the addition of an aqueous suspension of $Ca(OH)_2$ and the precipitated $CaSO_4$ removed and extracted twice with boiling water. The $SO_4^{2-}$ remaining in the combined filtrate and washings was removed with $Ba(OH)_2$. $Ca^{2+}$, still in solution, was removed with oxalic acid, the small precipitate in each case being collected and washed on the filter with boiling water. The hydrolysate, freed from oxalate, $SO_4^{2-}$, $Ca^{2+}$ and $Ba^{2+}$, was then concentrated *in vacuo* to about 0·5 l. and filtered to remove any insoluble material. To the filtrate (about 700 ml.) solid powdered copper picrate (395 g., 20 % in excess of double the amount calculated to combine with the glycine present in the ratio of 1 mole of picrate to 2 moles of amino acid) was added and brought into solution by gentle warming. On cooling to room temp., yellowish green crystals of the glycine–copper picrate complex salt began to separate, until after a few hours the mixture had turned into a heavy solid mass of crystals. After 2 days at 0° the crystalline ppt. was filtered off, sucked dry and then washed with 100 ml. of 5 % copper picrate solution in two portions. The product so obtained was recrystallized from a large volume of hot water. After keeping the soln. overnight at 0° the well-crystallized complex was collected at the pump, washed with a little ice-cold water and finally dried in a vacuum desiccator over $H_2SO_4$. The combined mother liquor and washings from the recrystallized compound, on concentration *in vacuo* to a small volume, yielded a small crop of similar crystals. The total yield was 190 g.

Glycine–copper picrate complex contains 6 moles of water of crystallization, which are lost at 110°. (Found: water of crystallization, 13·95. Calc. for $(C_2H_5O_2N)_2$, $Cu(C_6H_2O_7N_3)_2,6H_2O$, 13·88 %. Found: C, 28·41; H, 2·16; N, 16·93; Cu, 9·37. Calc. for $(C_2H_5O_2N)_2,Cu(C_6H_2O_7N_3)_2$: C, 28·67; H, 2·11; N, 16·73; Cu, 9·49 %.)

*Properties of the glycine–copper picrate complex.* The new compound crystallizes rapidly from hot aqueous soln. in the form of shining yellowish green crystals which appear under the microscope as transparent flat plates. On heating the dry crystalline hydrate at 100–120°, a dark green compound is obtained which is the anhydrous form of the glycine–copper picrate complex. When the compound is allowed to crystallize from a small volume of boiling water two different crystalline substances are obtained. A dark-green picrate which crystallizes in clusters of needles separates faster than a light-green picrate which crystallizes as large individual plates. The dark green compound was found to be the basic form of the glycine–copper picrate complex, whereas the light-green substance was the original neutral complex. The filtrate from the two compounds, on concentration *in vacuo*, yields a mixture of glycine picrate and picric acid.

Decomposition of the glycine–copper picrate complex into the basic derivative was brought to completion by treating the finely powdered substance (15 g.) with refluxing ethanol for 15 min. On cooling to room temp., the resulting dark-green precipitate was collected, washed with a little ice-cold water and finally dried at 110° (7·2 g.). It was far less soluble in water than the original compound. Analysis showed that it was the basic form of glycine–copper picrate complex salt. The yield corresponds to 99·6% of the theoretical. (Found: N, 14·72; Cu, 16·95. Calc. for $(C_2H_5O_2N)_2,Cu(C_6H_2O_7N_3)_2,CuO$: N, 14·95; Cu, 16·97%.) The filtrate and washings from this operation were combined, concentrated *in vacuo* to dryness and the residue (5·73 g.) was repeatedly extracted with ether. The combined ether extracts, on evaporation to dryness, yielded 2·16 g. of pure picric acid, equivalent to 98% of the theoretical amount. The residue remaining after the removal of picric acid was taken up in the minimum volume of boiling water and the soln. filtered to remove any insoluble substance. After chilling the filtrate for 12 hr. the bright yellow crystalline picrate which had separated was collected, washed with a little ethanol and recrystallized from a small volume of boiling water. The resulting product had m.p. 198–199°, which is characteristic of pure glycine picrate (Cox & King, 1929).

*Isolation of glycine.* The glycine–copper picrate complex (150 g.) was heated with 700 ml. 10% (v/v) $H_2SO_4$ on a steam-bath for 1 hr. with frequent stirring and shaking. The liberated picric acid was removed from the mixture by extraction with successive portions of hot benzene and $Cu^{2+}$ was removed from the acid aqueous solution by $H_2S$ as CuS. The resulting solution was decolorized by boiling for several minutes with a little acid-washed charcoal and was then completely freed from $H_2SO_4$ with $Ba(OH)_2$. The filtrate and washings from $BaSO_4$ were concentrated *in vacuo* to a syrup and glycine was precipitated with ethanol. After standing overnight at 0° glycine was collected and washed with ethanol. It was recrystallized by dissolving it in the minimum amount of boiling water and precipitating it with ethanol. The recrystallized product weighed 26 g. and when heated slowly decomposed at 230–235°. A sample (0·5 g.) was converted into hippuric acid. The recrystallized hippuric acid had m.p. 192–193°, which was not depressed on admixture with an authentic specimen, m.p. 192–193°.

*Isolation of glycine–copper picrate complex from egg-albumin hydrolysates*

Egg albumin (100 g.) was hydrolysed with $H_2SO_4$ as described above. The hydrolysate was diluted with twice its volume of water and humin filtered off. The filtrate was decolorized by boiling for 5 min. with 20 g. of charcoal. To the combined filtrate and washings $Ca(OH)_2$ in slight excess of the $H_2SO_4$ present was added, and the $CaSO_4$ removed and washed. The free ammonia was removed by partial concentration of the filtrate *in vacuo*. $SO_4^{2-}$ and $Ca^{2+}$ were completely removed in the manner previously described and the solution was concentrated *in vacuo* to about 200 ml. The small amount of amino acids which had separated was removed and washed several times with ice-cold water. The combined filtrate and washings (about 300 ml.) were treated with 200 g. of copper picrate, gently warmed until all the solid had dissolved, and then cooled. After 3 days at 0° the mixture was filtered and the glycine–copper picrate complex washed with a little ice-cold water. The product was recrystallized as described above (12·5 g.). Analysis confirmed that it was pure glycine–copper picrate complex.

## SUMMARY

1. A new method for the isolation of glycine from protein hydrolysates by its direct precipitation with copper picrate is described.

2. The method has the advantages that it is simple and economical in time and reagents. The glycine–copper picrate complex is readily decomposed by acids, and picric acid can easily be recovered.

3. The introduction of undesirable reagents is avoided, and after the removal of glycine the hydrolysate is left in a form suitable for the isolation of other amino acids.

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# Quantitative Determination of the Different Higher Saturated Fatty Acids in Fat from Blood, Chyle and Faeces, by Means of Partition Chromatography on Rubber

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In the study of fat metabolism, it is important to examine the behaviour of each constituent fatty acid of the ingested fat under investigation. For this purpose a method is necessary by which a quantitative determination of each of the different saturated and unsaturated fatty acids can be carried out, not only in the fat ingested but also in the fat of the blood, chyle and faeces. Reversed-phase partition chromatography according to Boldingh (1950, 1953) was found to be very satisfactory for the analysis of the higher saturated fatty acids. With this method it is possible to obtain an accurate quantitative analysis of a mixture of 10 mg. of each of the higher saturated fatty acids from hexanoic to tetracosanoic.

A description is given in this article of the quantitative determination of the different saturated fatty acids in blood serum, chyle and faeces. The blood-fat analysis has been carried out with blood fat from normal healthy adults. The other data have been chosen from an investigation of the fat in the chyle of a patient with a chylothorax after the consumption of various fats (Fernandes, Kamer & Weijers, 1955), and from an investigation of faecal fat in patients with coeliac disease and pancreatic fibrosis, whose diet contained different fats during different experimental periods (Weijers & Kamer, in preparation).

## METHODS AND RESULTS

The procedure adopted for the estimation of the higher fatty acids in blood, chyle and faeces consists of the following steps: extraction of the fats; saponification and removal of unsaponifiable material; extraction of the total fatty acids with light petroleum; removal of the unsaturated fatty acids by oxidation with $KMnO_4$; separation and estimation of the saturated fatty acids by the chromatographic method of Boldingh (1950, 1953). Removal of the unsaturated acids was essential, since their presence interfered with the separation of the saturated fatty acids. An estimate of total unsaturated fatty acids present is obtained by determination of the iodine value of the fatty acids present in the extract, using a separate sample of the extract. The widely differing nature of the materials to be analysed, i.e. blood, chyle and faeces, necessitated different methods of extraction.

### *Chromatographic method*

*Apparatus.* Cylindrical tubes (total length about 45 cm.; internal diameter 10·75–10·85 mm.) with side tube, provided with separating funnel of 200 ml. (see Fig. 1).

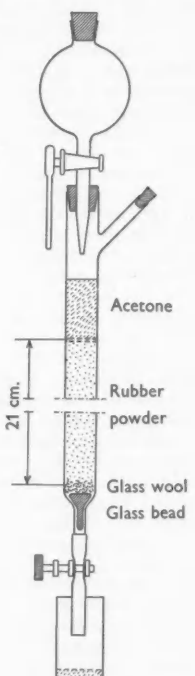


Fig. 1. Cylindrical tube containing the rubber column according to Boldingh (1950, 1953) to separate the saturated fatty acids.

Chrome-nickel sieve plates, diameter about 10·8 mm., with about 250 holes per cm.² (a square perforation of 0·3 mm.). Cylindrical tubes of 50 ml. calibrated at 20 ml. Fig. 2 shows the arrangement for titration under a nitrogen atmosphere.

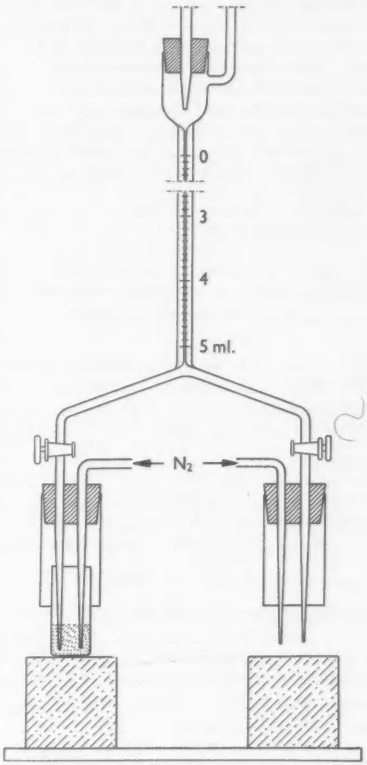


Fig. 2. Double arrangement for microtitration under a nitrogen atmosphere.

*Solvents.* Acetone, distilled over anhydrous $K_2CO_3$. Acetone 30% (v/v); 600 ml. $CO_2$-free distilled water was added to 300 ml. acetone in a 1 l. measuring cylinder, mixed, and made up to 1 l.

Acetone 45, 55, 60, 65, 70, 74, 77 and 80% (v/v) was prepared in the same way as acetone 30% (v/v).

Neutral arachis oil. Arachis oil was purified by chromatography on $Al_2O_3$: the $Al_2O_3$ (Brockmann) was heated for 6 hr. at 600°. After cooling, 4% water (w/w) was added and the system thoroughly mixed and 80 g., suspended in light petroleum, was used for preparation of the column (diam. about 2·5 cm.), arachis oil (100 g.) was dissolved in 200 ml. light petroleum and this solution was allowed to run through the column. The light petroleum was completely removed from the eluate by distillation *in vacuo* at 100°, under a stream of nitrogen.

Sodium ethoxide solution, 0·005 N, was prepared by dissolving metallic sodium in 96% purified ethanol.

*Preparation of the rubber suspension.* The rubber powder was prepared from 'Mealorub' ('Rubberstichting', Delft, The Netherlands) as follows: Rubber (150 g.) was washed well by rinsing in water and dried by suction through a Büchner filter. The rubber was then boiled for 1 hr. with 450 ml. acetone and 50 ml. 25% (w/w) HCl. Treatment of the rubber with HCl served to remove the ZnO added for vulcanization, which otherwise interferes with the separation of the fatty acids. The rubber was filtered, boiled again for 1 hr. after adding acetone and HCl, and this treatment was repeated twice more. Finally, it was boiled twice for 1 hr. with acetone only, after which it was washed with acetone until free from acid (litmus paper) and dried by suction. The rubber was then suspended in acetone and kept at least 2 days and at most 1 month. Before grinding, the acetone was filtered off from a portion of the rubber and light petroleum added until the acetone was displaced. Next, the rubber, just moist with light petroleum, was ground for 4 hr. in a ball mill half-filled with balls of different sizes; we used a vibratory Ball Mill (Griffin and Tatlock R.P.M. 1425) with a porcelain pot of 1 l. The mill must be tightly closed so that no light petroleum evaporates. The rubber powder so obtained was sieved, whilst stirring under light petroleum, through a sieve with pores of 0·3 mm. (British Standards Institution (1943) 410, mesh no. 52). It is important that the rubber should not be too fine (a quantity of rubber sufficient for 5·5 columns should pass the sieve in about 15 min.).

Sufficient of the milled and sieved rubber suspension in light petroleum is poured into a measuring cylinder, so that after settling for about 15 min., the volume of the rubber is 177 ml. (this quantity is sufficient for 5 columns, viz. 5 × 32 ml. with a surplus of half a column). The light petroleum is then decanted, the rubber transferred quantitatively to a Büchner funnel and washed with acetone until completely free from light petroleum. Neutral oil (19 ml.) is pipetted into an Erlenmeyer flask, and the rubber added with the aid of 126·5 ml. acetone and mixed well, after which 91 ml. water is added drop by drop whilst swirling. The rubber suspension thus obtained is allowed to stand overnight.

*Preparation of the rubber column.* The chromatographic tube, which was closed at the lower end by rubber tubing with screw clamp, was half filled with 65% acetone and a glass bead dropped in and covered with a layer (0·5 cm. thick) of finely cut glass wool which was pressed well down. The acetone was then run off until it was 1 cm. above the glass wool, and the screw clamp was closed again. The tube was then practically filled with the rubber suspension (see above) and the clamp opened and the tube rotated in as nearly vertical a plane as possible. In order to avoid warming the column with the hand, the constricted lower end of the tube was rotated between thumb and forefinger, while the uppermost part of the tube was held in place by a small metal ring in which it was able to revolve freely. Fresh rubber suspension was gradually added to the tube (which must never be allowed to run dry) until the rubber column was 21 cm. high. Care was taken that the level of the acetone was 5 cm. above the rubber. A chrome-nickel sieve plate was placed on the column. With the aid of a long glass rod flattened at the tip, the column of rubber was compressed to a height of 19 cm., the screw clamp was closed and the rubber particles floating in the acetone above the chrome-nickel sieve plate were removed by means of a pipette with rubber bulb. Next the column was

washed with about 50 ml. 65 % acetone, care being taken to prevent the column from running dry. The column was then washed with about 50 ml. 30 % acetone which caused it to shrink about 0·5–1·0 cm. A wet cloth was put round the tube in order to keep the temperature below that of the atmosphere, thus preventing the formation of gas bubbles in the column (cf. Pikaar, 1955).

*Addition of acids to the column.* The rubber tubing with the screw clamp was removed and the 30 % acetone was run off until the level was just beneath the sieve plate. Meanwhile, 1 ml. of the solution of fatty acids in acetone–oil (~5–10 mg. saturated fatty acids) was pipetted and transferred drop by drop to the sieve plate on the rubber column. Care was taken that after adding one drop, the liquid fell to just under the sieve plate before adding the following drop, regulating this by opening and closing the tube underneath with a finger, care being taken that the rubber did not run dry. After the fatty acid solution had been added, three 0·5 ml. portions of 30 % acetone were added, and when these had run in, the tube was filled with 30 % acetone. The tube was then closed with the rubber tubing and screw clamp, and the separating funnel, filled with 140 ml. 30 % acetone, was placed on the tube.

*Elution and titration.* The rate of flow was adjusted to about 20 ml./15 min. and seven 20 ml. fractions were collected. Nitrogen (purified by washing with alkali, acid and water) was passed through for 3 min. and each fraction was titrated with 0·005 N sodium ethoxide under a stream of nitrogen. The sum of the titrations is $a_1$ ml. Next the separating funnel was filled with 140 ml. 45 % acetone and seven fractions of 20 ml. each were collected and titrated, giving a total titration of $a_2$ ml. This procedure was repeated with 55, 60, 65, 70, 74, 77 and 80 % acetone. The blank titration of each of the acetone mixtures was determined by titrating 20 ml. samples with 0·005 N ethanolic alkali, giving $b_1$, $b_2$, $b_3$, ... ml.

To check the sum of the titrations of the different fractions obtained by elution, 1 ml. of the original fatty acid solution in acetone–oil was titrated, after 7 ml. acetone and 3 ml. 96 % ethanol had been added (*c* ml.). This value, corrected for a blank titration of 7 ml. acetone and 3 ml. 96 % ethanol (*d* ml.), has to be equivalent to

$$(a_1+a_2+a_3+\ldots)-7(b_1+b_2+b_3+\ldots) \text{ ml.}$$

For calculation the following formula is used:

$$\text{mg. fatty acid per 140 ml.} = (a-7b)\times N_{\text{alkali}} \times \text{mol.wt.}$$

The molecular weights of the fatty acids, corresponding with the different acetone concentrations are summarized in Table 1.

Table 1. *Molecular weights of fatty acids, eluted by different acetone concentrations*

| Acetone (%, v/v) | Fatty acid | Mol.wt. |
|---|---|---|
| 30 | Hexanoic + octanoic | 130 |
| 44 | Decanoic | 172 |
| 55 | Dodecanoic | 200 |
| 60 | Tetradecanoic | 228 |
| 65 | Hexadecanoic | 256 |
| 70 | Octadecanoic | 284 |
| 74 | Eicosanoic | 312 |
| 77 | Docosanoic | 340 |
| 80 | Tetracosanoic | 368 |

Sometimes 7 fractions are not sufficient for a complete elution; in such a case eight or nine times 20 ml. must be collected until the value of the blank titration is (approximately) reached.

In order to make the separation of the different fatty acids more complete, 10 ml. instead of 20 ml. can be collected at the beginning and end of an elution, if desired.

If the analysis has to be interrupted during the night, it is best to stop the analysis after a certain elution has been completed. The following day an elution is first carried out once with 20 ml. of the elution liquid last used. A considerable titration value is then found, especially in the case of the higher acetone concentrations, which, however, is not caused by higher fatty acids, so that it must be discarded. Next the elution is continued in the usual way with seven times 20 ml. of the next elution liquid.

The recovery of fatty acids from a mixture of pure materials is shown in Table 2.

Table 2. *Percentage recovery of saturated fatty acids from a known mixture*

| Fatty acids | Amount weighed (mg.) | Recovery (mg.) | Recovery (%) |
|---|---|---|---|
| Hexanoic acid and octanoic acid | 5·0 / 5·0 | 9·4 | 94 |
| Decanoic acid | 5·0 | 4·8 | 96 |
| Dodecanoic acid | 5·0 | 4·9 | 98 |
| Tetradecanoic acid | 5·0 | 4·75 | 95 |
| Hexadecanoic acid | 5·0 | 5·0 | 100 |
| Octadecanoic acid | 5·0 | 4·9 | 98 |

### *Analysis of blood*

*Reagents.* Ethanol–ether mixture (3 vol. ethanol/1 vol. peroxide-free ether). N Sodium ethoxide was prepared by dissolving metallic Na in 96 % ethanol which had been purified by boiling for 1 hr. with solid NaOH, followed by distillation and storage in the refrigerator.

Quinol, 0·1 % in 96 % ethanol; this solution should be colourless. Light petroleum, b.p. 60–80°, was washed twice with 33 % NaOH, followed by washing twice with water; dried with $MgSO_4$,2·5$H_2O$ and then distilled after addition of about 5 g. neutral arachis oil/4 l. light petroleum. Magnesium sulphate, $MgSO_4$.2·5$H_2O$ ($MgSO_4$.7$H_2O$ is gently heated until a loss of weight of 32–35 % has occurred). Ethanol, 96 % by volume; ethanol, 75 % by volume. Thymol blue solution, neutralized 0·2 % in 96 % ethanol.

*Extraction, saponification and purification.* The lipids from serum were extracted according to Bloor (1914), by adding 25 ml. serum to 750 ml. of an ethanol–ether mixture (3:1), drop by drop with continual swirling. The mixture is boiled for 1 min., filtered through defatted filter paper and washed quantitatively with hot ethanol–ether. N Sodium ethoxide (10 ml.) and 0·5 ml. quinol solution were added to the filtrate, the ethanol

and ether distilled off and the remainder evaporated to dryness on the water bath under nitrogen. The residue so obtained was dissolved in 10 ml. sodium ethoxide (care being taken that all was dissolved), 0·5 ml. of the quinol solution was then added and the mixture evaporated to dryness on the boiling-water bath while introducing nitrogen above the surface for 15 min. An absolutely dry white crust is left. This crust was taken up in about 10 ml. water, 36 ml. 96 % ethanol was added and the mixture extracted three times with 25 ml. light petroleum; the light petroleum extracts were shaken once with 75 % ethanol. The ethanolic phases were combined and about 12 ml. 4N-HCl (red to thymol blue) and 90 ml. water were added. This solution was extracted three times with 50 ml. light petroleum; most of the light petroleum was distilled off from the pooled extracts and sufficient 0·1N sodium ethoxide was added to give a blue colour, using thymol blue as indicator, and this solution was evaporated to dryness.

*Removal of the unsaturated fatty acids.* The dry residue obtained as described above was dissolved in 20 ml. water (the solution should be clear). To this was added 1 ml. 33 % (w/w) KOH and mixed well, if necessary with gentle heating. After cooling, $KMnO_4$ solution was added until the colour of the solution remained just visibly purple after standing for 15 min.; normally 5–10 ml. were required. The solution was set aside for 1 hr. at room temp. and then acidified with about 4 ml. 50 % (w/v) $H_2SO_4$ and the excess $KMnO_4$ removed by the gradual addition of solid $NaHSO_3$. After addition of 96 % ethanol up to a concentration of about 40 % the solution was extracted three times with light petroleum and the extract washed with 20 ml. weakly acidified (HCl) water in order to remove the sulphuric acid, and the light petroleum was evaporated quantitatively. The dry residue was then dissolved in exactly 5 ml. acetone containing 20 % (v/v) neutral arachis oil. The saturated fatty acids in 1 ml. of this solution were determined according to the chromatographic procedure described above.

*Results.* Table 3 gives the analysis of the blood fat of a normal adult after fasting for 12 hr.

Hence, the blood fat of this subject contained 1 % dodecanoic acid ($C_{12}$), 2 % tetradecanoic acid ($C_{14}$), 20 % hexadecanoic acid ($C_{16}$) and 6 % octadecanoic acid ($C_{18}$); the remaining 72 % was unsaturated fatty acids.

From the titrated values for dodecanoic, eicosanoic and docosanoic acid the corresponding amounts of fatty acid have not been calculated, because these single determinations are too small to allow a conclusion to be drawn as to the presence of these fatty acids.

Table 4 gives the analysis of the blood fat of a normal adult without and with addition of known quantities of fatty acids.

Table 3. *Saturated fatty acids in the blood of a normal adult*

Fatty acids were extracted as described in the text and unsaturated acids removed by oxidation with $KMnO_4$; 19·7 mg. of the total fatty acids were taken and after oxidation with $KMnO_4$ were equivalent to 5·91 ml. 0·005N-NaOH.

| Fatty acids ... ... ... | | | Dodecanoic acid | Tetradecanoic acid | Hexadecanoic acid | Octadecanoic acid | Eicosanoic acid | Docosanoic acid |
|---|---|---|---|---|---|---|---|---|
| Concentration of acetone in the developing solvent (% v/v) ... | 30* | 45* | 55 | 60 | 65 | 70 | 74 | 77 |
| Total of titrations (ml. 0·005N-NaOH) | 1·18 | 0·39 | 0·39 | 0·62 | 3·28 | 1·07 | 0·38 | 0·40 |
| Total of blanks (ml. 0·005N-NaOH) | 0·21 | 0·20 | 0·24 | 0·28 | 0·32 | 0·28 | 0·28 | 0·21 |
| Difference | 0·97 | 0·19 | 0·15 | 0·34 | 2·96 | 0·79 | 0·10 | 0·19 |
| Fatty acids in mg. | — | — | 0·15 | 0·39 | 3·8 | 1·12 | — | — |
| Fatty acids as % of fat in the blood | — | — | 1 | 2 | 20 | 6 | — | — |

* These fractions contain the oxidation products of the unsaturated acids and the titrations cannot, in consequence, be equated with hexanoic, octanoic and decanoic acids, which are, in any case, attacked by $KMnO_4$—see Table 5.

Table 4. *Percentage recovery of saturated fatty acids added to blood fat*

| Fatty acids | Found in blood fat (mg.) | Added to blood fat (mg.) | Found in blood fat after addition (mg.) | Recovery of added fatty acids (mg.) | Recovery of added fatty acids (%) |
|---|---|---|---|---|---|
| Tetradecanoic acid | 0·61 | 3·20 | 4·00 | 3·37 | 106 |
| Hexadecanoic acid | 5·61 | 2·79 | 8·60 | 2·99 | 107 |
| Octadecanoic acid | 1·44 | 4·40 | 5·84 | 4·40 | 100 |

*Analysis of chyle*

The fat is extracted from chyle, using the method described by Röse-Gottlieb (cf. Williams, 1950, p. 456) for the extraction of fat from milk, by adding ammonia and ethanol and extracting by shaking with a mixture of equal parts of ether and light petroleum.

*Apparatus and reagents.* Weighing bottles diam. 1·5 cm., height 1·5 cm.; sodium ethoxide, 0·1 N. For other reagents, see Analysis of Blood.

*Extraction, saponification and purification.* To 100 g. chyle in a separating funnel were added 20 ml. 10 % $NH_4OH$, 100 ml. 90 % ethanol, 250 ml. ether and 250 ml. light petroleum in this order. The mixture was shaken after each addition and finally shaken vigorously for 1 min. After 1 hr. the ether–light petroleum layer is collected and the ammoniacal ethanol layer again shaken for 1 min. with a mixture of 250 ml. ether and 250 ml. light petroleum. The ether and the light petroleum were distilled off from the combined extracts. When fatty acids which are easily oxidized are present the work is carried out in an atmosphere of nitrogen. About 2 g. of the fat residue were weighed out and saponified by boiling for 30 min. under reflux with 25 ml. 0·5 N potassium ethoxide. Water (7 ml.) was added and after cooling the mixture was shaken twice with 20 ml. light petroleum. The collected light petroleum extracts were shaken once with 5 ml. 75 % ethanol. The combined ethanolic solutions were acidified with 10 ml. 4 N-HCl and then 64 ml. water were added. This was shaken twice with 75 ml. light petroleum and the light petroleum was distilled off from the combined extracts.

*Removal of unsaturated fatty acids.* About 125 mg. of the fatty acids were weighed out, dissolved in 10 ml. 96 % ethanol and made alkaline with about 4 ml. 0·1 N ethanolic alkali. The ethanol from the alkaline soap solution was evaporated quantitatively. The residue was dissolved in 20 ml. water, 1 ml. 33 % KOH was added and the solution treated as described for blood.

Besides the higher saturated fatty acids chyle fat, contrary to blood fat, may also contain octanoic and decanoic acids. When these acids are to

Table 5. *Oxidation of the saturated fatty acids by* $KMnO_4$

Percentage recovery of 100 mg. fatty acids after 1 hr. treatment with excess of $KMnO_4$ at 20°

| Fatty acid | Recovery (%) |
|---|---|
| Octanoic acid | 77 |
| Decanoic acid | 96 |
| Dodecanoic acid | 99 |
| Tetradecanoic acid | 100 |
| Hexadecanoic acid | 100 |
| Octadecanoic acid | 100 |

Table 6. *Estimation according to Boldingh's technique of the saturated fatty acids of chyle fat in a chylothorax patient after ingestion of coconut oil*

| | Without oxidation with $KMnO_4$ (25·9 mg. of fatty acids, iodine value 37·5) | | | After oxidation of the unsaturated fatty acids with $KMnO_4$ (27·1 mg. of fatty acids ~21·0 ml. of 0·005 N-NaOH) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acids ... ... ... | Hexanoic + octanoic acid | Decanoic acid | Dodecanoic acid | — | — | Dodecanoic acid | Tetra-decanoic acid | Hexa-decanoic acid | Octa-decanoic acid | Eicosanoic acid |
| Concentration of acetone of the elution liquids in % (v/v) ... | 30 | 45 | 55 | 30 | 45 | 55 | 60 | 65 | 70 | 74 |
| Total of titrations in ml. 0·005 N-NaOH | 0·42 | 0·94 | 9·31 | 3·18 | 1·13 | 9·39 | 4·04 | 3·54 | 1·03 | 0·36 |
| Total of blanks | 0·35 | 0·35 | 0·35 | 0·35 | 0·35 | 0·35 | 0·21 | 0·21 | 0·21 | 0·21 |
| Difference | 0·07 | 0·59 | 8·96 | 2·83 | 0·78 | 9·04 | 3·83 | 3·33 | 0·82 | 0·15 |
| Fatty acids in mg. | — | 0·5 | 9·0 | — | — | 9·0 | 4·4 | 4·3 | 1·2 | — |
| Fatty acids as % of fat in the chyle | — | 2 | 35 | — | — | 33 | 16 | 16 | 4 | — |

be determined, the $KMnO_4$ oxidation for the removal of the unsaturated fatty acids cannot be applied since both octanoic and decanoic are attacked by this treatment, see Table 5.

It is a lucky circumstance that the unsaturated fatty acids do not interfere with elution of these two acids and hence they can be determined without $KMnO_4$ treatment.

In practice, a suitable amount of fatty acids is straight away dissolved in 5 ml. acetone–oil and, without oxidation, the chromatographic analysis is carried out with 1 ml.

*Results.* Table 6 gives the analysis of chyle fat from a chylothorax patient who had eaten a meal containing coconut oil.

Table 7 gives the analysis of chyle fat of the same patient after addition of known quantities of some fatty acids.

triglycerides present in the first light petroleum extract were saponified with potassium ethoxide. After the unsaponifiable substances had been removed by shaking with light petroleum in alkaline medium, the solution was acidified and the fatty acids derived from the original triglycerides present were extracted with light petroleum. For the rest the procedure was the same as that described for the analysis of the total fatty acids.

*Reagents.* Ethanol, 75%, alkaline to thymol blue (pH > 9); hydrochloric acid 1N in 25% ethanol. For other reagents, see Analysis of Blood.

*Extraction, saponification and purification.* Fresh faeces (20 g., corresponding to at least 200 mg. saturated fatty acids) were boiled with 160 ml. ethanol (containing 0·4% amyl alcohol) and 40 ml. 33% KOH for 20 min. under reflux. After cooling, about 50 ml. light petroleum were added and the

Table 7. *Percentage recovery of saturated fatty acids added to chyle fat*

| Fatty acids | Found in chyle fat (mg.) | Added to chyle fat (mg.) | Found in chyle fat after addition (mg.) | Recovery of added fatty acids (mg.) | Recovery of added fatty acids (%) |
|---|---|---|---|---|---|
| Dodecanoic acid | 2·17 | 3·98 | 6·43 | 4·26 | 107 |
| Tetradecanoic acid | 1·11 | 3·38 | 4·52 | 3·41 | 101 |
| Hexadecanoic acid | 1·00 | 3·36 | 4·46 | 3·46 | 103 |
| Octadecanoic acid | 0·32 | 3·58 | 3·68 | 3·36 | 94 |

*Analysis of faeces*

Faeces contain free fatty acids, soaps and triglycerides, and it is possible to determine either the total amount of each fatty acid present in these three forms or to determine the amounts present in each form separately.

For the estimation of the total fatty acids present as free acids, soaps and triglycerides, the faeces were boiled with potassium ethoxide and the ethanolic extract was treated with light petroleum to remove the unsaponifiable material. The fatty acids present in the ethanolic extract were liberated by acidification, extracted with light petroleum and purified by treating the light petroleum extract with 25% (w/v) aqueous ethanol (Kamer, Bokkel Huinink & Weijers, 1949). The unsaturated fatty acids were removed by oxidation and the saturated acids separated and estimated chromatographically as described above.

For the estimation of the free fatty acids and soaps and the triglycerides separately, the faeces were boiled with acid, made alkaline and, after addition of ethanol to a concentration of 75%, the triglycerides were extracted with light petroleum. The ethanolic lower layer was acidified and, the fatty acids derived from the free fatty acids and the soaps were extracted with light petroleum. The mixture was shaken for 1 min. The clear light petroleum layer was separated from the ethanolic layer and the latter was shaken again for 1 min. with 50 ml. light petroleum. If the light petroleum extract had not immediately cleared, it was left to stand overnight, or filtered. The collected light petroleum extracts were shaken once with about 15 ml. 75% ethanol, and the light petroleum layers discarded. The ethanolic phases were acidified with 60 ml. 25% (w/v) HCl, and the ethanol concentration was brought to 60% (v/v) by adding water; after this the mixture was shaken three times for 1 min. with about 75, 50 and 50 ml. light petroleum respectively. The light petroleum extracts were purified by shaking once with about 85 ml. 25% ethanol in N-HCl (van de Kamer, 1953) and the light petroleum was distilled off.

Sometimes an extra purification of the faeces extract was advantageous. It is possible to combine this purification with the oxidation of the unsaturated fatty acids by oxidizing with $KMnO_4$ during the night instead of for 1 hr. However, it is necessary to take into account not only a loss of hexanoic, octanoic and decanoic acid, but also a slight loss of dodecanoic acid (3%).

*Removal of unsaturated fatty acids.* For the removal of the unsaturated fatty acids about 100 mg. of the mixed fatty acids were weighed out,

Table 8. *Estimation of the saturated fatty acids in the faeces of a patient with coeliac disease*

Unsaturated fatty acids removed by oxidation with $KMnO_4$; 34·5 mg. of fatty acids were equivalent to 22·7 ml. 0·005N-NaOH.

| Fatty acids ... ... ... | | | Dodecanoic acid | Tetradecanoic acid | Hexadecanoic acid | Octadecanoic acid | Eicosanoic acid | Docosanoic acid |
|---|---|---|---|---|---|---|---|---|
| Concentration of acetone of the elution liquids in % (v/v) ... | 30 | 45 | 55 | 60 | 65 | 70 | 74 | 77 |
| Total of titrations } ml. 0·005N-NaOH | 1·47 | 0·52 | 0·45 | 2·31 | 11·09 | 7·32 | 0·44 | 0·48 |
| Total of blanks } | 0·21 | 0·28 | 0·21 | 0·21 | 0·29 | 0·21 | 0·24 | 0·24 |
| Difference | 1·26 | 0·24 | 0·24 | 2·10 | 10·80 | 7·11 | 0·20 | 0·24 |
| Fatty acids in mg. | — | — | — | 2·4 | 13·8 | 10·1 | — | — |
| Fatty acids as % of the fat in faeces | — | — | — | 7 | 40 | 29 | — | — |

Table 9. *Percentage recovery of saturated fatty acids added to faecal fat*

| Fatty acids | Found in faecal fat (mg.) | Added to faecal fat (mg.) | Found in faecal fat after addition (mg.) | Recovery of added fatty acids (mg.) | Recovery of added fatty acids (%) |
|---|---|---|---|---|---|
| Decanoic acid | — | 3·88 | 3·88 | 3·88 | 100 |
| Dodecanoic acid | 0·77 | 2·19 | 2·88 | 2·11 | 96 |
| Tetradecanoic acid | 0·74 | 3·25 | 4·32 | 3·58 | 110 |
| Hexadecanoic acid | 3·84 | 2·13 | 6·05 | 2·21 | 104 |
| Octadecanoic acid | 2·47 | 4·12 | 6·77 | 4·30 | 104 |

dissolved in 96 % ethanol, made alkaline, evaporated to dryness and taken up in about 20 ml. water (clear) and treated further as described for the analysis of blood.

*Results.* Table 8 gives the analysis of fat in faeces from a coeliac patient whose diet contained butter and rice flour.

Owing to the low fat content of faeces, viz. 4 %, the faecal extract contained a relatively large amount of compounds other than fatty acids with the result that the separation of the fractions eluted with 60, 65, and 70 % acetone respectively was not as precise as usual. Therefore only 0·14 ml. of the overnight increase of 0·38 ml. was neglected from the calculations and it was assumed that the remaining 0·24 ml. corresponded with tetradecanoic acid; but it is not impossible, however, that a portion of this corresponds with hexadecanoic acid. These uncertainties have only slight influence on the percentage composition of the original fatty acid mixture.

Altogether 14·45 ml. were titrated; the titration of the same amount of fatty acid before the analysis was 14·57, so that 99 % was recovered.

Table 9 gives the analysis of faecal fat without and with addition of known quantities of some fatty acids.

## SUMMARY

The extraction, purification and quantitative determination of the saturated fatty acids in blood, chyle and faeces are described. In order to carry out the quantitative determination, it is necessary to remove the unsaturated fatty acids by oxidation with $KMnO_4$ in an alkaline medium. The quantitative determination of the saturated fatty acids hexanoic acid ($C_6$), octanoic acid ($C_8$) and decanoic acid ($C_{10}$) and of the unsaturated $\Delta^{13}$-docosanoic acid ($C_{22}$) before oxidation with $KMnO_4$, and of the saturated fatty acids dodecanoic acid ($C_{12}$), tetradecanoic acid ($C_{14}$), hexadecanoic acid ($C_{16}$), octadecanoic acid ($C_{18}$), eicosanoic acid ($C_{20}$) and docosanoic acid ($C_{22}$) after oxidation with $KMnO_4$, is carried out by means of reversed-phase partition chromatography according to Boldingh (1950, 1953).

Some examples of the application are given.

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# Two Steps in the Reaction of Chymotrypsin with Acetyl-L-Phenylalanine Ethyl Ester

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Studies of the effect of the hydrogen-ion concentration on the catalytic activity of various enzymes have long been recognized as a powerful tool for the exploration of the nature of the active sites as well as of the mechanisms involved in such reactions (Dixon, 1953; Alberty, 1954; Alberty, Massey, Frieden & Fuhlbridge, 1954; Wilson, 1954). One of us (Gutfreund, 1955) has found that the ionization constant of the catalytic site of the enzyme could be determined accurately for the trypsin-catalysed hydrolysis of benzoyl-L-arginine ethyl ester. Moreover, owing to a number of fortunate properties of that system, there is less ambiguity about the properties and chemical nature of the catalytic site of trypsin than there is about other enzymes which have been studied in a similar way. However, neither in the case of trypsin nor in that of the chymotrypsin system to be described here is the chemical nature of the ionizing catalytic group identified beyond any doubt, though one can say that the imidazole group of histidine would have all the necessary properties. Apart from a study of the catalytic site the present investigation was undertaken to compare the reaction mechanism of chymotrypsin with that of trypsin. For trypsin Gutfreund (1955) found that the reaction of the enzyme with its substrate involves two separate steps: first, an initial fast adsorption at the specificity site; and secondly, a slow, rate-determining step involving a reaction of the catalytic site with the carbonyl groups of the substrate. In the trypsin-catalysed reaction the enzyme–substrate combination was shown to be independent of the subsequent reaction at the catalytic site and similar conclusions can be drawn from the results of experiments with chymotrypsin to be described here. Earlier work on the chymotrypsin-catalysed hydrolysis of derivatives of phenylalanine and tyrosine has been reviewed by Neurath & Schwert (1950).

## EXPERIMENTAL

The procedure for following the rate of ester hydrolysis used in this laboratory has been described recently (Bernhard, 1955). One minor modification was the use of a magnetic stirrer. Instead of bubbling nitrogen through the reaction mixture it was circulated in the space above it. Under these conditions $CO_2$-free water did not change its pH during prolonged stirring. All reactions were carried out in a water bath maintained at $25 \pm 0{\cdot}05°$.

Crystalline α-chymotrypsin was obtained from Worthington Biochemicals Corporation, Freehold, N.J. For each day's experiments an enzyme solution was made up by 500-fold dilution in water from a single stock solution containing about 0·5% enzyme protein in 0·1 M-$NaH_2PO_4$–$Na_2HPO_4$ buffer at pH 5·0. One ml. of this enzyme solution was added to 14 ml. of a solution of appropriate pH, buffer and substrate concentration. The reaction mixture was 0·1 M with respect to NaCl.

The substrate, acetyl-L-phenylalanine ethyl ester, was the same sample used and described by Bernhard (1955).

It was found that tenfold changes in the concentration of three buffer systems (0·005–0·05 M) used (diethylamine, glycine and ethylenediaminetetraacetic acid) had no inhibitory or accelerating effect on the hydrolysis reaction catalysed by chymotrypsin, and thereafter glycine was used as buffer at pH 8 and ethylenediaminetetraacetic acid at all other pH values.

## RESULTS

The reaction scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

(where E, S, ES and P are the enzyme, substrate, enzyme–substrate complex and product respectively) leads to the kinetic expression for the velocity $v$,

$$v = k_3[E]\,[S]/(K_m + [S]) \qquad (1)$$

The results are expressed in the form of the pH dependence of $K_m$ and of $v_0$, where $v_0$ is the initial velocity at concentration of substrate $[S_0]$. Values for $K_m$ were obtained by plotting $1/v_0$ against $1/[S_0]$ (Lineweaver & Burk, 1934), the value of $1/[S_0]$ at $1/v_0 = 0$ being equal to $-1/K_m$, as shown in Fig. 1 for measurements on the system chymotrypsin–acetyl-L-phenylalanine ethyl ester at pH 7.

The values for $K_m$ over the range of pH 6·5–9 are given in Fig. 2 and a plot of $k_3[E]$ against pH is given in Fig. 3; $k_3[E]$ was calculated from experiments at $[S_0] = 1{\cdot}97 \times 10^{-3}$ M by Eqn. 1. $k_3[E]$ is in arbitrary units, since only relative values are required for our conclusions. A calculation of the absolute rate of hydrolysis of acetyl-L-phenyl-

alanine ethyl ester at 25° and pH 7·8, based on the molecular weight of chymotrypsin $M = 23\,000$, gives the value $k_3 = 160$ sec.$^{-1}$.

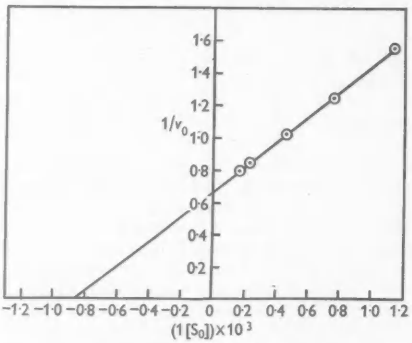


Fig. 1. Determination of $K_m$ for the reaction of chymotrypsin with acetyl L-phenylalanine ethyl ester at pH 7 and 25°. $K_m$ and the initial substrate concentration $[S_0]$, are in moles/l. The initial velocity, $v_0$, is in arbitrary units.

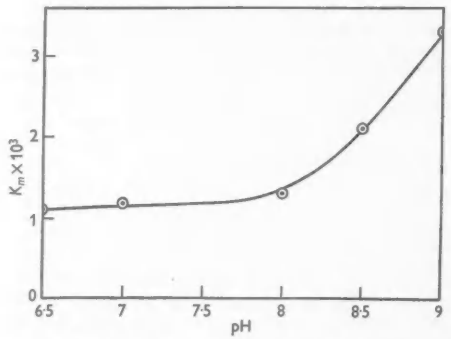


Fig. 2. Variation of $K_m$ with pH for the reaction between chymotrypsin and acetyl L-phenylalanine ethyl ester at 25°. $K_m$ is in moles/l.

## DISCUSSION

The Michaelis constant $K_m = (k_2 + k_3)/k_1$ as defined by Briggs & Haldane (1925) approximates to the true equilibrium constant $K_m = [E]\,[S]/[ES]$ if $k_3 \ll k_2$. Gutfreund (1954) has pointed out that for the chymotrypsin-catalysed hydrolysis of acetyl-L-phenylalanine ethyl ester $k_3$ is small compared with $k_2$. The results reported here, which show that $K_m$ is constant from pH 6·5 to 8·0, while $k_3[E]$ changes fourfold over this pH range, are consistent with $K_m$ being the true equilibrium constant of enzyme–substrate binding.

The Michaelis–Menten equation, which involves only one form of the enzyme–substrate complex, is an over-simplification but it is adequate for the derivation of the kinetics for the formation and decomposition of the rate-determining complex. It follows from the results of the effect of pH on the maximum velocity $k_3[E]$ that hydrogen ions act as non-competitive inhibitors of chymotrypsin-catalysed hydrolysis reactions. An analytical description of the general principle of such inhibitions has been given by Botts & Morales (1953). One can conclude from this that the reaction at the specific

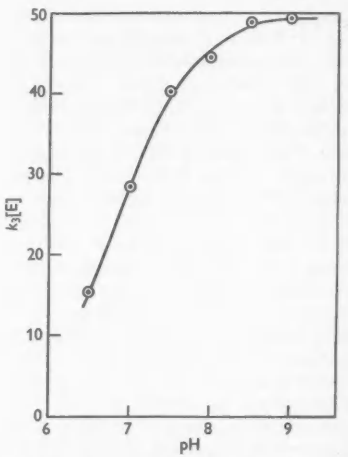


Fig. 3. Variation of the maximum velocity, $k_3[E]$ (in arbitrary units) with pH for the reaction between chymotrypsin and acetyl L-phenylalanine ethyl ester at 25°.

site of the enzyme, resulting in the formation of the initial enzyme–substrate complex characterized by $K_m$, is independent of the subsequent reaction of the substrate with the catalytic site of the enzyme. We can therefore expand the Michaelis–Menten equation as follows

$$E + S \underset{}{\overset{k_1}{\rightleftharpoons}} (ES)_1 \overset{k_3}{\rightleftharpoons} (ES)_2 \rightarrow E + P.$$

The rate of enzyme–substrate complex formation is very large, $k_1 > 10^6$ l. M$^{-1}$ sec.$^{-1}$ (Gutfreund, 1954), while $k_3$, the rate-determining formation of a labile chemical intermediate compound $(ES)_2$, is 100–200 sec.$^{-1}$, for various chymotrypsin preparations. A more detailed picture as well as the analogy of this second step with the nucleophilic attack of an electron donor on the carbonyl carbon during base-catalysed ester hydrolyses has been given by Gutfreund (1955).

The ionization constant of the catalytic site can be determined either by the method used by

Gutfreund (1955) or by the determination of $K_i$, the inhibition constant,

$$K_i = [E][H^+]/[EH^+],$$

where [E] is the concentration of active enzyme and $[EH^+]$ the concentration of inhibited enzyme. From the equations given by Lineweaver & Burk (1934) one can get the following relation between inhibitor concentration $[H^+]$, $k_3[E]$ the maximum velocity at a particular $[H^+]$ concentration and $k_3[E_0]$ the maximum velocity when all enzyme present is in the active form

$$[E_0]/[E] = 1 + [H^+]/K_i.$$

The ratio $[E_0]/[E]$ at different values of $[H^+]$ was calculated from determinations of $k_3[E]$, taking $k_3[E_0] = k_3[E]$ at pH 9·0. The dissociation constant $K_i = 1{\cdot}42 \times 10^{-7}$ and $pK_i = 6{\cdot}85$ for the catalytic site of chymotrypsin has been calculated from the slope of the plot of $[H^+]$ against $[E_0]/[E]$ given in

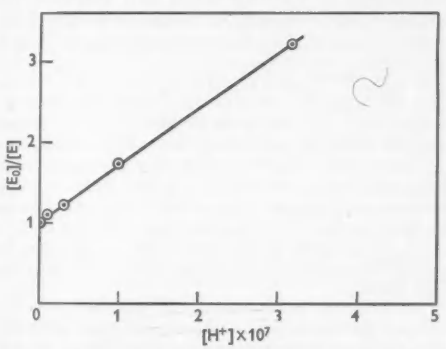


Fig. 4. Determination of the inhibition constant, $K_i$ (in moles/l.) for the non-competitive inhibition of chymotrypsin by hydrogen ions. The slope of the graph is $1/K_i$.

Fig. 4. In the case of trypsin hydrolysing benzoyl-L-arginine ethyl ester $pK_i$ (= 6·25) was found to be constant in the presence of phosphate, *p*-nitrophenol and ethylenediaminetetraacetic acid as buffer (unpublished observations). Sri Ram, Terminiello, Bier & Nord (1954) have studied the pH dependence of the casein hydrolysis by trypsin and by acetylated trypsin. Approximate $pK_i$ values can be determined from their data and come to 6·25 for trypsin, a very basic protein, and 7·10 for acetyltrypsin, an acidic protein. Such a shift of the p*K* of the catalytic site of the acetylated enzyme to a more alkaline pH is to be expected. Similarly, since chymotrypsin is a less basic protein than trypsin, the p*K* of the catalytic site should be at a more alkaline pH in the case of chymotrypsin even if the sites are chemically identical in the two enzymes. Further evidence for the identification of the imidazole group of histidine as the catalytic site of chymotrypsin was summarized by Desnuelle (1954). Model reactions of imidazole derivatives were studied by Wagner-Jauregg & Hackley (1953) and the inactivation of chymotrypsin during photo-oxidation was described by Weil & Buchert (1952). Though all the evidence as to the chemical nature of the catalytic group is necessarily only circumstantial, the known physical properties and chemical reactivity of imidazole would account for the behaviour of the catalytic site.

An acyl migration from histidine to serine might form part of the over-all reaction (Desnuelle, 1954). The above results would be consistent with such an additional step, provided that all steps taking place after the formation of $(ES)_2$ are fast compared with the rate of formation of $(ES)_2$.

## SUMMARY

1. The effect of pH on $k_3$, the first-order rate constant for the decomposition of the enzyme–substrate complex, and on $K_m$, the Michaelis constant, has been studied for the chymotrypsin-catalysed hydrolysis of acetyl-L-phenylalanine ethyl ester.

2. The results presented are consistent with our previous finding that $K_m$ is the true dissociation constant $E + S \rightleftharpoons ES$ and that this initial rapid enzyme substrate combination is independent from the subsequent catalytic step.

3. Evidence for the chemical nature of the catalytic site of chymotrypsin is summarized and it is suggested that it is the imidazole group of histidine.

B.R.H. is a Hackett student of the University of Western Australia and H.G. is an Imperial Chemical Industries Research Fellow of the University of Cambridge.

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# The Effect of Some Pantothenate Derivatives on Growth and Coenzyme-A Synthesis in Bacteria

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As shown previously (Pierpoint & Hughes, 1952, 1954) the synthesis of coenzyme A (CoA) by washed suspensions of *Lactobacillus arabinosus* is greatly stimulated by the addition of cystine. Because pantetheine replaces both pantothenate and cystine it seems probable that cystine gives rise to the β-mercaptoethylamine residue of pantetheine and hence of CoA. In order to study further the role of cystine in CoA synthesis a number of pantothenyl peptides related to pantetheine have been tested for their ability to replace pantothenate or pantothenate and cystine for the growth of some bacteria and for CoA synthesis in washed suspensions of *Lb. arabinosus*.

## MATERIALS AND METHODS

*Organisms.* *Lactobacillus arabinosus* 17-5 (National Collection of Industrial Bacteria no. 8014), *Leuconostoc mesenteroides* P 60 (N.C.I.B. no. 6990) and *Proteus morganii* (National Collection of Type Cultures, nos. 2818, K 653 and a locally isolated strain, KI) were maintained by daily subculture on the casein hydrolysate–glucose medium of Nossal (1951). *Lactobacillus helveticus* Strain 80 was a culture obtained from Dr E. E. Snell; it was maintained on the above medium enriched by the addition of pantetheine ($10^{-6}$ M).

*Growth tests.* The ability of the various pantothenate derivatives to replace pantothenate for the growth of *Lb. arabinosus* was tested on the pantothenate assay medium of Barton-Wright (1952). The medium (4 ml.) was placed in 6 in. × ½ in. test tubes, covered with aluminium caps (Oxoid Ltd.). Growth was measured turbidimetrically in an EEL nephelometer (Evans Electroselenium Ltd., Harlow, Essex) after incubation at 37° for 16–18 hr. In some experiments the lactic acid formed was also measured by titration with 0·02 N-NaOH in a Beckman automatic titrator. The results obtained by the two methods agreed satisfactorily. The substances to be tested were either sterilized by filtration through a Knight & Fildes (1936) type Seitz filter and added to the medium aseptically, or autoclaved at 10 lb./in.² for 10 min. after addition to the medium. Growth tests with *Lb. helveticus* were carried out as above except that the test medium was that of Craig & Snell (1951), to which was added Tween 80 (Honeywill and Stein Ltd., 21 St James's Square, London, S.W. 1) in the place of Tween 40 and oleic acid. The medium for *P. morganii* was that of Barton-Wright (1952) for cystine assay, in which the salts *A* and the sodium acetate were replaced by 4·5 g. $KH_2PO_4$ and the pH was adjusted to 7·6. Tests were carried out in 50 ml. conical flasks to which side-arm test tubes had been sealed. This was to allow an adequate supply of oxygen while the medium was incubated in the flasks; growth could then be measured by tipping the medium into the side arm which fitted the nephelometer.

The effects of pantothenate and pantetheine on the cystine requirements of *Lb. arabinosus* and *Ln. mesenteroides* were tested on the cystine-assay medium (Barton-Wright, 1952).

*The synthesis of CoA by washed suspensions.* The ability of compounds to replace pantothenate, or, in compounds containing sulphur, to replace pantothenate and cystine, was tested with washed suspensions of pantothenate-deficient cells as previously described (Pierpoint & Hughes, 1954). Washed cells (approx. 10 mg.) were suspended in saline to which was added 0·05 M phosphate buffer (pH 7·0) and glucose (0·05 M). Pantothenate and cysteine when added

$HO.CH_2.CMe_2.CH(OH).CO.NH.CH_2.CH_2.CO.R$
General formula of pantothenyl peptides

| Compound | R |
|---|---|
| Pantetheine | $-NH.CH_2.CH_2.SH$ |
| Pantothenylcysteine | $-NH.CH(CO_2H).CH_2.SH$ |
| Pantothenylserine | $-NH.CH(CO_2H).CH_2.OH$ |
| Pantothenyl-2-hydroxyethylamine | $-NH.CH_2.CH_2.OH$ |
| Pantothenylaminoacetaldehyde | $-NH.CH_2.CHO$ |
| Pantothenylglycine | $-NH.CH_2.CO_2H$ |
| Pantothenyl-α-alanine | $-NH.CHMe.CO_2H$ |
| Pantothenyl-β-alanine | $-NH.CH_2.CH_2.CO_2H$ |
| Pantothenamide | $-NH.H$ |
| Pantothenylglutamic acid | $-NH.CH(CO_2H).CH_2.CH_2.CO_2H$ |

Fig. 1. Structures of pantothenyl derivatives.

were at a concentration of $2 \times 10^{-4}$M and the various pantothenate derivatives were added over a range of $10^{-5}$ to $10^{-3}$M. The suspensions were incubated at 37° for 60 min., after which the cells were collected and washed by centrifuging. Their CoA content was estimated by the method of Handschumacher, Mueller & Strong (1950). The results are expressed as Lipmann units CoA. Where weights of organisms are given these refer to dry weights and were estimated by drying a sample of the washed suspension at 110° and subtracting the dry weight of an equal volume of saline.

*Materials.* The derivatives of D(+)-pantothenate (Fig. 1) were synthesized by Baddiley & Mathias (1954). The amino acids were commercial samples and, with the exception of L(+)-glutamic acid, were not purified further. The glutamic acid was contaminated with cystine and histidine, from which it was freed by passage through Zeo-Karb 215, elution with HCl and crystallization (Partridge & Westall, 1949). No cystine was detected in the product by either microbiological or chromatographic tests.

## RESULTS

### *Effect of pantothenate derivatives on the synthesis of CoA by washed suspensions*

Pantetheine-4′-phosphate, which supports CoA synthesis in liver (Levintow & Novelli, 1952), did not support synthesis in washed suspensions of *Lb. arabinosus*. Pantothenyl-L-cysteine, pantothenyl–cysteine-L-4′-phosphate and dipantothenyl-L-cystine gave rise to 0–10 % of the amount of CoA given by equivalent amounts of pantothenate and cystine, but this effect was variable and may have been due to traces of impurities in the samples.

The following pantothenyl peptides, which do not contain sulphur (Fig. 1), were found to be without appreciable activity in replacing pantothenate for synthesis when cystine was also present: *N*-pantothenyl-2-hydroxyethylamine, pantothenyl-DL-α-alanine, pantothenyl-DL-serine, pantothenyl-β-alanine, pantothenyl-DL-glutamic acid, *N*-pantothenylaminoacetaldehyde and pantothenamide. In contrast to this pantothenylglycine replaced pantothenate to some extent (Fig. 2), but was never as active as pantothenate itself. The effectiveness of different samples of the compound was the same in any single batch of organisms but varied widely in different batches of cells (Table 1). Although the

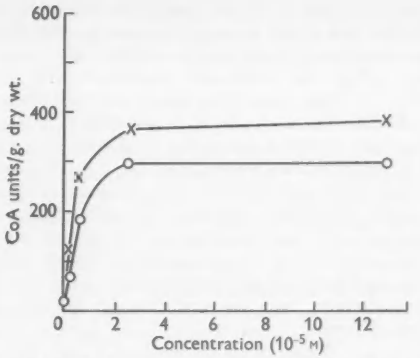


Fig. 2. The effect of varying concentrations of pantothenylglycine and pantothenate on CoA synthesis. Deficient cells (6·4 mg.) were suspended in glucose–phosphate–saline containing cystine ($2 \times 10^{-4}$M) and either pantothenic acid (×) or pantothenylglycine (o). They were incubated at 37° for 60 min. and their CoA contents estimated.

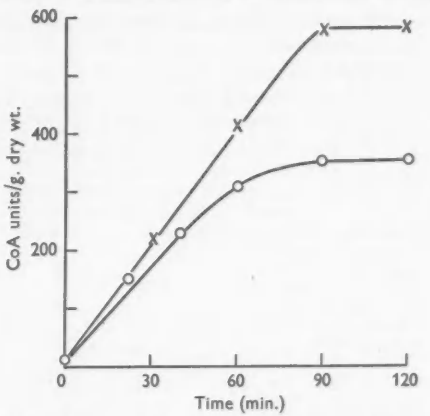


Fig. 3. Time curve of CoA synthesis from pantothenylglycine and pantothenate. Washed deficient cells (9·3 mg.) were suspended in 1·9 ml. phosphate saline containing glucose, $1{\cdot}6 \times 10^{-4}$M cystine and either $10^{-4}$M pantothenylglycine (o) or $10^{-4}$M pantothenic acid (×).

Table 1. *CoA synthesis from pantothenylglycine and pantothenate*

The organisms were suspended in the phosphate saline containing glucose, $2 \times 10^{-4}$M cystine, and either $10^{-4}$M pantothenylglycine or $10^{-4}$M pantothenate. The suspensions were incubated at 37° for 60 min. and the CoA was then estimated.

| Expt. | Organism (mg./tube) | CoA synthesized from pantothenylglycine (units/tube) Sample 1 | Sample 2 | Sample 3* | Synthesis (percentage of that from pantothenate) |
|---|---|---|---|---|---|
| 1 | 10 | 1·7 | 1·8 | — | 53 |
| 2 | 9 | 0·6 | 0·6 | — | 18 |
| 3 | 6·4 | — | 2·15 | — | 77 |
| 4 | 5·5 | — | 1·3 | 1·4 | 54 |

* Sample 3 was the dimethylamine salt.

initial rate of CoA synthesis from pantothenylglycine was often almost the same as that from pantothenate, the synthesis came to a stop sooner and before the cells were saturated with CoA (Fig. 3). The lack of activity of pantothenylglutamate is of interest, since King, Fels & Cheldelin (1949) reported the formation of a very active pantothenate compound by yeasts on incubation with pantothenate and glutamic acid. In view of the present findings it is possible that their glutamic acid was contaminated by cystine, which commonly occurs in most samples of glutamic acid prepared from natural sources, and that the active pantothenate was pantetheine or one of its derivatives.

### *Effect of pantothenate derivatives on the growth of* Lb. arabinosus

Of the pantothenate peptides not containing sulphur, only pantothenylglycine had marked activity in replacing pantothenate for the growth of *Lb. arabinosus* (Table 2). The results of growth

Table 2. *Effect of pantothenyl derivatives in replacing pantothenate as a growth factor for* Lb. arabinosus

The compounds were tested in concentrations from $10^{-7}$ to $2 \times 10^{-5}$ M. Growth was measured turbidimetically at intervals up to 100 hr.

| Compound | Growth-promoting activity relative to pantothenate (%) |
|---|---|
| *N*-Pantothenyl-2-hydroxyethylamine | 5 |
| Pantothenyl-L-cysteine | 1 |
| Dipantothenyl-L-cystine | 3 |
| Pantothenyl-DL-α-alanine | 1 |
| Pantothenyl-β-alanine | 1 |
| Pantothenyl-DL-serine | 1 |
| Pantothenamide | 2 |
| *N*-Pantothenylaminoacetaldehyde | 1 |
| Pantothenylglycine | 80–100 |
| Pantetheine-4′-phosphate | 1 |
| Pantothenyl-L-glutamic acid | 6 |

Pantothenylaminoacetaldehyde increased some 28% in activity when autoclaved in the medium. None of the other compounds showed a similar change in activity.

tests thus agree with the experiments on washed suspensions. Although after about 16 hr. growth promoted by pantothenylglycine was considerably less than that promoted by equivalent amounts of pantothenate, at 60 hr. it was the same (Fig. 4). This lag before maximum growth is attained is similar to the effect of pantetheine described by Craig & Snell (1951). Under the present conditions, however, pantetheine would only support 10–25% of the growth produced by pantothenate (Fig. 5); similar findings have also been reported with this organism by McRorie & Williams (1951).

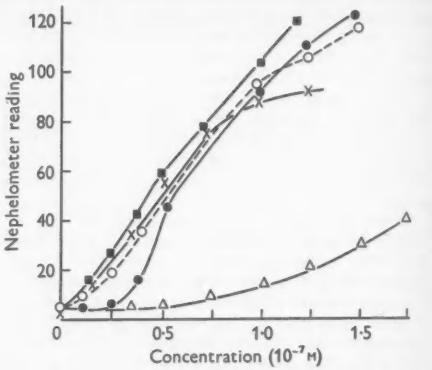


Fig. 4. Replacement of pantothenate by pantothenylglycine for the growth of *Lb. arabinosus* 17-5. Growth in the presence of pantothenylglycine was measured at 17 hr. (△), 42 hr. (●) and 66 hr. (○- - -○). In the presence of pantothenate, it was measured at 17 hr. (×), 42 and 66 hr. (■).

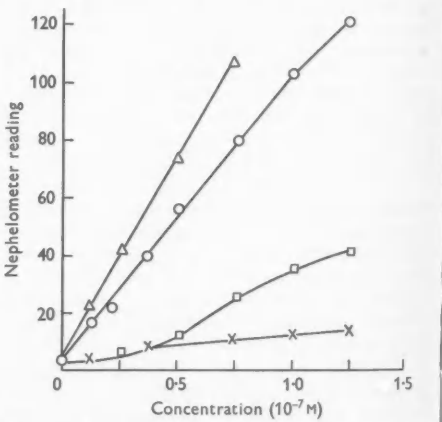


Fig. 5. Replacement of pantothenate by pantetheine for the growth of *Lb. arabinosus*. Growth supported by pantothenic acid was measured at 16 hr. (○) and 130 hr. (△), and by pantetheine at 16 hr. (×) and 130 hr. (□). The results were not affected by the method used to sterilize the pantetheine.

### *Effect of pantothenate derivatives on the growth of* Lb. helveticus

In this organism, pantetheine is approximately 100 times more active in promoting growth than is pantothenate (Fig. 6) (cf. Craig & Snell, 1951). When tested in concentrations up to $10^{-5}$ M the following compounds were without marked effect in

replacing pantetheine: pantothenyl-L-cysteine, di-pantothenyl-L-cystine, pantothenyl-DL-serine, pantothenyl-L-cysteine-4′-phosphate, pantetheine-4′-phosphate, pantothenyl-β-alanine, pantothenyl-DL-α-alanine, pantothenylglycine, pantothenamide.

*Effect of pantothenate derivatives on the growth of* P. morganii

Pantetheine had approximately the same activity as pantothenate in supporting the growth of *P. morganii* (Table 3). The compounds listed above were tested with one strain (K 653) and only pantothenyl-β-alanine had any marked growth-promoting activity, amounting to 20–30 % of the equivalent effect of pantothenate after 3 days' growth.

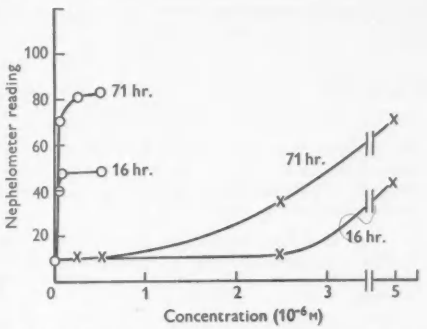


Fig. 6. The effect of pantetheine (○) and pantothenic acid (×) on the growth of *Lb. helveticus*.

Table 3. *Effect of pantetheine and pantothenate on the growth of* P. morganii (*strain K* 653)

Conditions were as described in the text, and growth was measured after 15 hr.

| Concn. of growth factor ($10^{-6}$ M) | Nephelometer reading: Pantothenate present | Nephelometer reading: Pantetheine present |
|---|---|---|
| 0 | 4 | 6 |
| 5 | 46 | 52 |
| 25 | 59 | 61 |
| 250 | 108 | 109 |

*Effect of pantoyltaurine on the utilization of pantoylglycine*

The activity of pantothenylglycine in supporting growth and CoA synthesis in *Lb. arabinosus* suggests either that it is an intermediate in the synthesis or that it is hydrolysed and thus forms a source of pantothenate. Attempts were made to detect the formation of glycine upon incubation of the peptide with washed suspensions. These were unsuccessful owing to release by the cells into the medium of large amounts of amino acids, including glycine. The effect of pantoyltaurine, which is known to inhibit pantothenate utilization (McIlwain & Hughes, 1944), was therefore tested. If pantothenylglycine were indeed an intermediate in CoA synthesis it would be expected to be much more active than pantothenate in overcoming inhibition by pantoyltaurine. However, both in growth tests (Table 4), and in experiments on CoA synthesis by washed suspensions (Table 5),

Table 4. *Effect of pantoyltaurine on the growth of* Lb. arabinosus

The organism was grown on the pantothenic acid assay medium (Barton-Wright, 1952) with the addition of the growth factors listed below. The Anti-Bacterial Index, or the ratio of the molar concentration of inhibitor to the molar concentration of growth factor necessary to produce complete inhibition of growth, was measured after 4 days' incubation at 37°.

| Expt. | Growth factor | Concn. of pantoyltaurine ($10^{-4}$ M) | Anti-Bacterial Index |
|---|---|---|---|
| 1 | Pantothenate | 1·3 | 6000 |
| | Pantothenylglycine | 1·7 | 1000 |
| | Pantetheine | 9 | 1000 |
| 2 | Pantothenate | 1·7 | 6000–10 000 |
| | Pantothenylglycine | 1·7 | 1000 |
| | Pantetheine | 8·6 | 1000 |

Table 5. *Effect of pantoyltaurine on the synthesis of CoA from pantoylglycine and pantothenate in* Lb. arabinosus

Pantothenate-deficient cells were washed and suspended in phosphate saline containing glucose and 0·28 M pantoyltaurine. They were incubated for 15 min. at 37°, and then cystine and either pantothenate or pantothenylglycine were added to final concentrations of $5 \times 10^{-3}$ M. The suspensions were further incubated for 60 min. and the CoA synthesized (units/tube) estimated.

| | | Expt. 1 | Expt. 2 |
|---|---|---|---|
| Organisms (mg./tube) ... ... | | 10 | 11 |
| CoA synthesized | From pantothenate | 3·9 | 2·5 |
| | From pantothenylglycine | 0·9 | 0·5 |
| CoA synthesized in presence of pantoyltaurine | From pantothenate | 3·2 | 1·4 |
| | From pantothenylglycine | 0·5 | 0·0 |
| Inhibition (%) | From pantothenate | 17 | 44 |
| | From pantothenylglycine | 45 | 100 |

pantothenylglycine was less effective in overcoming inhibitions by pantoyltaurine than was pantothenate. This result suggests that pantothenylglycine is hydrolysed to pantothenate before utilization for CoA synthesis in *Lb. arabinosus*. However, the correctness of this interpretation is doubtful since it was found that pantetheine too was less effective in overcoming inhibition by pantoyltaurine than was pantothenate. This is in contrast with its effect in some other organisms where it is more effective (McRorie & Williams, 1951).

### *Effect of pantothenate derivatives on the cystine requirements of* Lb. arabinosus

In order to follow the uptake of cystine from the medium by washed suspensions of *Lb. arabinosus*, estimations were made using the microbiological assay method of Barton-Wright with *Lb. arabinosus* as the test organism. It was found, however, that impossibly high recoveries of cystine were obtained when the mother-liquor in which cells had been incubated was tested. The addition of such a mother-liquor to the standards indicated that the error was due to a sparing effect at low concentrations of cystine; in the higher concentrations no effect on growth was found. Besides being needed for protein synthesis, cystine in this organism is also needed for CoA synthesis. It seemed possible therefore that some intermediate in the synthesis of CoA from pantothenate and cystine might have a sparing effect. As shown in Fig. 7 pantetheine had such an effect on the cystine requirements of *Lb. arabinosus*: that is, growth in the lower concentrations of cystine was increased several-fold by the addition of pantetheine, but no effect was found in the higher concentrations. No sparing effect was found with dipantothenyl-L-cystine, pantetheine-4′-phosphate and pantothenylglycine. Pantothenyl-L-cysteine had a small effect amounting to about 5% of that of equivalent amounts of pantetheine. High concentrations of pantothenate ($5 \times 10^{-4}$M) often delayed growth on the low concentrations of cystine but had no such effect in the higher concentrations (Fig. 7).

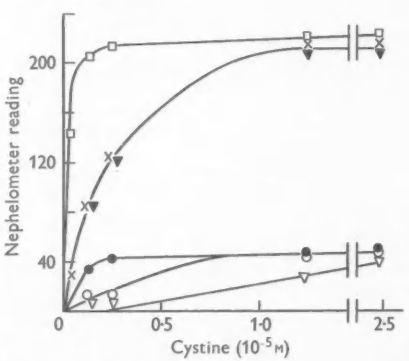


Fig. 7. The effect of pantetheine on the cysteine requirement of *Lb. arabinosus* 17·5. Growth was measured after incubation for 16 hr. (●) and 72 hr. (□) in the presence of $2{\cdot}5 \times 10^{-5}$M pantetheine. The control tubes were also measured after 16 hr. (○) and 72 hr. (×). Additional pantothenate was added ($5 \times 10^{-4}$M) and the growth read after 16 hr. (▽) and 72 hr. (▼).

Pantetheine had no sparing effect on the cystine requirements of *Ln. mesenteroides* (Table 6) and this may account for the greater specificity of the assay using this organism which has recently been described by Barton-Wright (1952).

Table 6. *Effect of pantetheine on the cystine-limited growth of* Ln. mesenteroides

The medium was that described by Barton-Wright (1952) and the growth conditions were the same as those used for *Lb. arabinosus*. When pantetheine was added, its final concentration was $2{\cdot}5 \times 10^{-5}$M.

| Cystine concn. ($10^{-5}$M) | Nephelometer reading pantetheine absent | | Nephelometer reading pantetheine present | |
|---|---|---|---|---|
| | 17 hr. | 36 hr. | 17 hr. | 36 hr. |
| 0·12 | 11 | 19 | 11 | 23 |
| 0·25 | 22 | 38 | 23 | 43 |
| 1·25 | 58 | 105 | 61 | 102 |
| 2·5 | 76 | 114 | 73 | 112 |

### *Uptake of cystine by washed suspensions of* Lb. arabinosus

Washed suspensions were incubated under the conditions described for CoA synthesis and the uptake of cystine was measured by microbiological assay using *Ln. mesenteroides* as the test organism. No interference with the assay was found with the incubation mother-liquor and satisfactory recoveries of cystine were obtained. The washed suspensions took up cystine from the incubation medium in both the presence and absence of pantothenate (Table 7). Cells which had been grown on the casein hydrolysate medium and in the presence of adequate pantothenate took up cystine but did not take up pantothenate. The cystine that was taken up in the absence of pantothenate could not be extracted from the dried cells by boiling with water for 10 min. There is thus no indication of the conversion of the cystine into a form which could be used for the synthesis of CoA on the addition of pantothenate; nor does this appear to occur during growth, because cells grown with adequate cystine, but with limiting amounts of pantothenate, still need the further addition of cystine for CoA synthesis (Pierpoint & Hughes, 1954).

Table 7. *Cystine uptake by washed cell suspensions*

Washed cells were incubated for 60 min. at 37° in the glucose–saline solution, with pantothenate and/or cystine in a final volume of 1·7 ml. After incubation the cells were collected and their CoA content was assayed. The pantothenate and cystine remaining in the supernatant liquid were then estimated.

| Expt. | Concn. of pantothenate in growth medium ($10^{-7}$M) | Organisms (mg./tube) | Cystine (μg.) | | | Pantothenate (μg.) | | | CoA (Lipmann units/tube) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Added | Remaining | Uptake | Added | Remaining | Uptake | Before incubation | After incubation | Synthesized |
| 1 | 1 | 12 | — | — | — | 2·93 | 1·22 | 1·7 | 0·55 | 1·17 | 0·62 |
| | | | 5·84 | 2·1 | 3·74 | 2·93 | 1·54 | 1·39 | 0·55 | 3·85 | 3·3 |
| | | | 5·84 | 2·1 | 3·74 | — | — | — | 0·55 | 0·67 | 0·12 |
| 2 | 1 | 9·8 | 9·6 | 4·55 | 5·05 | 6·9 | 3·55 | 3·35 | 0·1 | 4·55 | 4·45 |
| | | | 9·6 | 4·45 | 5·15 | — | — | — | 0·1 | 0·2 | 0·1 |
| 3 | 10 | 10 | — | — | — | 8·1 | 7·9 | 0·2 | — | 5·25 | — |
| | | | 9·7 | 7·8 | 1·9 | — | — | — | 5·4 | 5·4 | 0 |
| | | | 9·7 | 7·8 | 1·9 | 8·1 | 8·1 | 0 | 5·4 | 5·4 | 0 |

## DISCUSSION

The following provisional scheme for the synthesis of CoA from pantothenate has been suggested (Hughes, 1953; Novelli, 1953) from microbiological and enzymic studies:

(1) Pantothenate + cysteine → Pantothenylcysteine
(2) Pantothenylcysteine → Pantetheine + $CO_2$
(3) Pantetheine + ATP → Pantetheine-4′-phosphate + ADP
(4) Pantetheine-4′-phosphate + ATP → Dephospho-CoA + pyrophosphate
(5) Dephospho-CoA + ATP → CoA + ADP.

Steps (3), (4) and (5), by which pantetheine is converted into CoA, have been well characterized by isolation from liver and partial purification of the enzymes concerned (Levintow & Novelli, 1954; Hoagland & Novelli, 1954; Wang & Kaplan, 1954). The steps leading to the synthesis of pantetheine from pantothenate are less firmly established. Previously it seemed reasonably clear that cystine was in some way involved in the synthesis (Pierpoint & Hughes, 1954), and in the present paper additional but still rather indirect evidence supports this view. The additional evidence is that pantetheine has a sparing effect on the cystine requirements of *Lb. arabinosus*. This sparing effect is likely to be due to competition for cystine between processes using it for protein synthesis and processes using it for CoA synthesis when excess of pantothenate is added. When pantetheine replaces pantothenate all the cystine becomes available for protein synthesis, and growth in the presence of limiting cystine is greatly increased. Such a marked sparing effect of pantetheine is, however, somewhat surprising in view of its low activity as a growth factor for *Lb. arabinosus*. Recently, Brown & Snell (1954) have shown that the reduced forms of pantetheine and its derivatives are generally much more active as bacterial growth-factors than are the oxidized forms. It seems unlikely, however, that this is the explanation of the low activity of pantetheine as a growth factor in the present experiments because it was added to the growth medium as the SH compound and was autoclaved in the medium to ensure that it remained reduced at least until the medium was inoculated (also discussed by Cheldelin & King, personal communication).

The inactivity of pantothenylcystine and pantetheine-4′-phosphate in supporting the growth of lactobacilli and in supporting CoA synthesis in *Lb. arabinosus* is in agreement with the findings of Brown & Snell (1954). Since these compounds are active as growth factors for *Acetobacter suboxydans* the simplest explanation of their inactivity in the

other organisms is that they cannot enter the cells. This is a well-known limitation of microbiological methods for the study of synthetic sequences. An additional limitation, especially with growth tests and experiments with intact cells, is that compounds which are not normal intermediates may be converted into them by metabolic reactions. This is illustrated in the present work by the activity of pantothenylglycine, which may equal or even surpass that of pantetheine, although it is probably first converted into pantothenate. In view of such limitations in the microbiological approach to CoA synthesis the inclusion of pantothenylcysteine as an intermediate as shown in steps (1) and (2) is still hypothetical.

The main support for the inclusion of pantothenylcystine in the scheme is that it will serve as a growth factor for *Acet. suboxydans* and that dried cells of this organism will convert it into a substance which serves as a growth factor for *Lb. helveticus*, an organism which normally needs pantetheine for growth as shown by Brown & Snell (1953). In addition, these authors have shown that *P. morganii* converts pantothenate and cystine into a product that is without activity for *Lb. helveticus* until it is treated with dried cells of *Acet. suboxydans*. That this product is in fact pantothenylcystine, as suggested by the authors, cannot be accepted without further characterization, especially since both pantetheine-4′-phosphate and dephospho-CoA would behave in a similar fashion to pantothenylcystine in these microbiological tests (Brown & Snell, 1953). The formation of such phosphorylated compounds in bacteria is extremely likely because pantothenate itself is readily phosphorylated both in intact cells and cell-free extracts of *Lb. arabinosus* and yeasts (Baddiley, Hughes, Mathias & Pierpoint, 1954).

In animal tissues the status of pantothenylcysteine in the synthesis of CoA is likewise still uncertain. Hoagland & Novelli (1954) have shown that pantetheine may be formed by rat-liver preparations from pantothenylcysteine. In contrast to this, pantothenylcysteine cannot replace pantothenate for the growth of deficient chicks and rats (Thompson & Bird, 1954).

## SUMMARY

1. The following compounds have been tested for ability to replace pantothenic acid or pantetheine in growth and CoA synthesis by various bacteria: pantothenyl-L-cysteine; dipantothenyl-L-cystine; pantothenyl-L-cysteine-4′-phosphate; pantothenyl-β-alanine; pantothenyl-DL-α-alanine; pantothenyl-DL-serine; pantothenylglycine; *N*-pantothenylaminoacetaldehyde; *N*-pantothenyl-2-hydroxyethylamine; pantothenamide; pantothenyl-L-glutamic acid; pantetheine-4′-phosphate.

2. Of these substances only pantothenylglycine is an effective substitute for pantothenic acid in the growth of *Lb. arabinosus* and in CoA synthesis by suspensions of this organism. Evidence was obtained, by the use of pantoyltaurine as an inhibitor, that pantothenylglycine is effective only after its hydrolysis to pantothenic acid.

3. None of these compounds (pantothenyl-L-glutamic acid and *N*-pantothenyl-2-hydroxyethylamine were not tested) replace pantetheine in supporting the growth of *Lb. helveticus*.

4. With the exception of pantothenyl-β-alanine (pantothenyl-L-glutamic acid and pantothenyl-2-hydroxyethylamine were not tested), none of the above compounds supports the pantothenic acid-limited growth of *Proteus morganii*. This compound supports only 20–30% of the growth due to an equivalent amount of pantothenic acid.

5. Pantetheine has a sparing effect on the cystine requirements of *Lb. arabinosus* but not on those of *Leuconostoc mesenteroides*. This probably accounts, in part at least, for the greater reliability of the cystine assays in which the latter organism is used.

6. Washed suspensions of *Lb. arabinosus* took up cystine from the medium. The uptake was independent of the presence of added pantothenate or of the pantothenate concentration in the cells.

7. The mechanism of CoA synthesis is discussed in relation to these findings.

We wish to thank Professor H. A. Krebs for his interest in this work and Mr M. Clegg and Miss H. Marsden for technical assistance. This work was aided by a grant from the Rockefeller Foundation.

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# The Reduction of Diphosphopyridine Nucleotide by Ethanol under the Influence of X-Irradiation

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Two types of mechanism have been suggested to explain dehydrogenase action. On the one hand, it has been postulated that the oxidations and reductions may be univalent in character, with free radicals as intermediates (Waters, 1948; Swallow, 1953, 1954*a*; Barron & Levine, 1952). On the other hand, an ionic mechanism has been proposed (Burton & Kaplan, 1954). Recent work on the reduction of diphosphopyridine nucleotide (DPN) by sodium dithionite (Yarmolinsky & Colowick, 1954; Colowick, 1954; Swallow, 1955) supports the ionic mechanism.

In this paper are presented some further experiments on the reduction of DPN by ethanol under the influence of ionizing radiations, a reaction which is believed to proceed through the agency of free radicals. Some of the results have been briefly reported elsewhere (Stein & Swallow, 1954; Swallow, 1954*b*).

## EXPERIMENTAL

*Irradiation arrangements.* Two Victor Maximar X-ray sets were used. One was operated at 220 kvp and the radiation filtered through 1 mm. aluminium, while the other was operated at 190 kvp with the radiation unfiltered. Doses were determined by the $FeSO_4$ method (Miller & Wilkinson, 1952), the value for the yield being taken as 15·6 molecules of $Fe^{3+}$ produced/100 ev of energy absorbed (Farmer, Rigg & Weiss, 1955). The dose rate with the 220 kvp set was 640 r/min. and with the 190 kvp set 4000–5000 r/min. The temperature of irradiations was not controlled, and was normally 20–30°. Irradiation vessels were similar to those previously employed (Swallow, 1952), two sizes of tube being used, to take 1 or 3 ml. of solution.

Deoxygenation of solutions was by the method previously described (Swallow, 1952). When 3 ml. of solution were to be irradiated in the absence of dissolved oxygen, 1 ml. of water + volatile solute was first evaporated off by the deoxygenation procedure, and allowance made for changes in concentration. It was assumed that the rate of evaporation of each constituent was proportional to its vapour pressure and to its mole fraction in the liquid.

*Chemicals.* Oxidized DPN ($DPN^+$) was obtained from Sigma Chemical Co., U.S.A. (90% pure) and C. F. Boehringer and Soehne, Germany (100% pure). Reduced DPN (DPNH) was from Sigma Chemical Co. Pig-heart lactic dehydrogenase was kindly given by members of the Biochemistry Department, University of Cambridge. Crystalline yeast alcohol dehydrogenase was obtained from C. F. Boehringer and Soehne. Other chemicals were of A.R. purity.

*Determination of hydrogen.* This work was done at King's College, Newcastle upon Tyne, by kind permission of Dr J. Weiss. The author wishes to thank Dr R. G. A. Johnson, Mr G. G. Jayson and other members of the radiation chemistry group for help with the apparatus. Irradiations of 100 ml. solution (air-free) were performed with 200 kvp unfiltered X-rays at a dose rate of 3100 r/min. Gas analysis was by the modified method of Stein & Weiss (see Johnson, Scholes & Weiss, 1953) and results were accurate to within 10%.

*Ultraviolet absorption curves.* The curves shown in Figs. 1 and 2 were obtained by diluting the solution with 2 vol. of water and measuring optical densities with a Unicam SP. 500 spectrophotometer with distilled water as blank. Curves shown in Fig. 3 were on undiluted solutions.

## RESULTS

### *Absorption spectra*

The effect of X-irradiation on the absorption curve of oxygen-free aqueous solutions of $DPN^+$ (0·27 mM) containing ethanol (0·5 M) is shown in Fig. 1. This result was for solutions 0·01 M in sodium pyrophosphate, adjusted to pH 7·8 with HCl. It was found that the effect of a dose of 39000 r was the same when the buffer was 0·01 M borax–boric acid

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at pH 7·8, or 0·01 M borax–sodium hydroxide or 0·01 M sodium carbonate–sodium bicarbonate at pH 10·4. Attempts were made to irradiate at pH 12, but variable results were obtained. In some experiments there was an increase in optical density at 340 mμ., but in others only a strong increase at about 280–290 mμ. As $DPN^+$ itself is unstable in alkaline solution it was not considered desirable to investigate this point further.

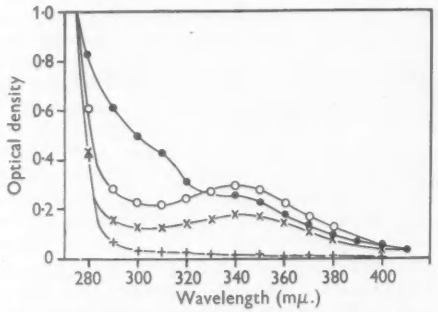


Fig. 1. Absorption curves of oxygen-free DPN solutions irradiated with X-rays in 0·5 M ethanol, pH 7·8. +, No irradiation; ×, dose, 39 000 r; ○, dose, 78 000 r; ●, dose, 156 000 r.

The substance absorbing at 340 mμ. is formed by the action of X- or γ-rays on $DPN^+$ under the same conditions as DPNH is formed by the action of alcohol dehydrogenase, except that for the formation of the irradiation product it is essential for oxygen to be absent (Swallow, 1953). As DPNH also absorbs at 340 mμ., it is natural to compare the two products. The full absorption curves of the two are shown in Fig. 2, together with the absorption curve of $DPN^+$. The irradiated solution still contains some unchanged $DPN^+$, and it is apparent that the absorption curve of the product is not very different from that of DPNH. Fig. 1 shows that the irradiation product changes in a characteristic fashion on prolonged irradiation, and it was also found that 0·27 mM DPNH in oxygen-free aqueous 0·5 M ethanol at pH 7·8 changed in the same way on X-irradiation with a dose of 117 000 r.

*Effect of adding acid to irradiated solutions*

It is known that when DPNH solutions are made acid, the absorption maximum at 340 mμ. disappears and is replaced by an increased ab-

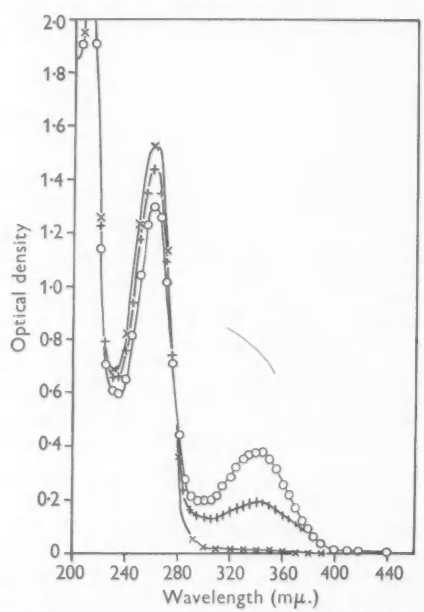


Fig. 2. Comparison of irradiation product with DPNH and $DPN^+$. ×, Absorption curve of 0·27 mM $DPN^+$; +, 0·27 mM DPN, oxygen-free, in 0·5 M ethanol, irradiated with a dose of 39 000 r; ○, 0·27 mM DPNH.

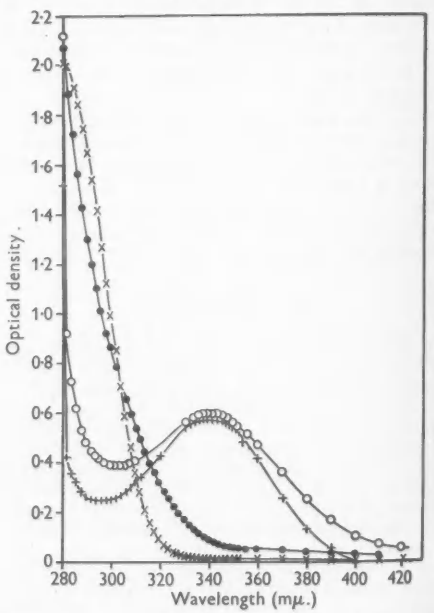


Fig. 3. Effect of adding dilute acid to reduced DPN solutions. +, DPNH, pH 7·8; ×, DPNH after acidification; ○, irradiated solution, pH 7·8; ●, irradiated solution after acidification.

sorption at 295 mμ. (Haas, 1936). Irradiated solution (3 ml.; in 0·01 M pyrophosphate buffer, pH 7·8) was acidified with 0·1 ml. N-HCl. DPNH solution (3 ml.) with the same initial optical density at 340 mμ. was similarly treated. The changes in absorption spectrum are recorded in Fig. 3. Addition of 0·1 ml. 10N- instead of N-HCl gave a similar change initially, but this was followed by a fairly rapid decrease in absorption at 290 mμ. in both cases, which made measurement of the absorption curve difficult. Apparently the substances which absorb at 290 mμ. are themselves attacked in strongly acid solutions.

*Reduction of methylene blue*

An important property of DPNH is that of reducing methylene blue to the colourless leuco form. Diaphorase increases the rate of decolorization, but was not used in the present experiments. 0·27 mM $DPN^+$ (3 ml.; pH 7·8) in oxygen-free 0·5 M aqueous, ethanol was irradiated with an X-ray dose of 39 000 r. A stream of nitrogen was then bubbled through at 23° for 20 min. to remove acetaldehyde. It was found that this solution would decolorize 0·05 ml. of mM methylene blue in 18 min. In a control experiment it was found that 1 ml. of 0·27 mM DPNH at pH 7·8 in 0·5 M ethanol would decolorize the same amount of methylene blue in 20 min.

*Fluorescence*

DPNH, in common with many other nicotinamide derivatives, has the property of fluorescing in the visible region of the spectrum under illumination with Wood's light (Sumner & Somers, 1947). It was found that 0·1 mM DPNH in aqueous 0·5 M ethanol at pH 7·8 exhibited an intense pale-green fluorescence, but a 0·1 mM $DPN^+$ in aqueous ethanol did not fluoresce. Solutions of the irradiation product, whether prepared at pH 7·8 or 10·2, exhibited only a barely detectable fluorescence. The lack of fluorescence was not due to quenching by acetaldehyde, for when a small quantity of the latter was added to a DPNH solution the fluorescence was unaffected. More conclusively, it was found that the addition of a small quantity of DPNH to an irradiated solution instantly produced the characteristic fluorescence. The lack of fluorescence proves that the irradiation product cannot be identical with DPNH.

*Coenzyme activity*

That the irradiation product differs from DPNH was confirmed by an experiment with lactic dehydrogenase. 0·27 mM $DPN^+$ (3 ml.) in oxygen-free aqueous 0·5 M ethanol at pH 7·8 was irradiated with an X-ray dose of 39 000 r. 10 mM Sodium pyruvate (0·1 ml.) was added, and, at $t = 0$, 0·1 ml. of a lactic dehydrogenase solution was added (Fig. 4). The optical density at 340 mμ. remained constant, showing that the enzyme was unable to catalyse the oxidation of the irradiation product by pyruvate. In order to prove that this could not be due to the presence of an enzyme inhibitor in the solution, a small amount of solid DPNH was added at $t = 15$ min. The optical density at 340 mμ. increased as a result of adding DPNH, and instantly began to decrease at the rate usual for pure DPNH solutions. The final pH of the solution was about 7·0, as the enzyme solution added was buffered to about this pH. Similar results were obtained for solutions irradiated at pH 10·2.

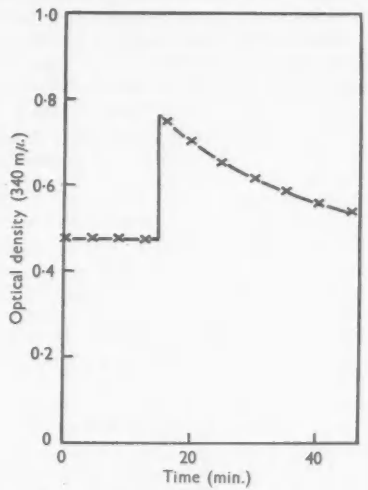


Fig. 4. Action of lactic dehydrogenase on a solution of the irradiation product containing sodium pyruvate. At $t = 0$ min. the lactic dehydrogenase was added, and at $t = 15$ min. DPNH was added.

A test with yeast alcohol dehydrogenase gave the same result. 0·36 mM $DPN^+$ (40 ml.) in aqueous, oxygen-free 0·46 M ethanol at pH 7·7 was irradiated with an X-ray dose of 45 000 r. When 3 ml. were placed in a spectrophotometer cuvette the optical density at 340 mμ. was found to be 0·77. Alcohol dehydrogenase solution (0·15 ml.) was added, and the optical density increased to 0·96, due to the enzyme-catalysed reduction of residual $DPN^+$ by ethanol. 0·5 M Acetaldehyde (0·15 ml.) was then added and the optical density found to decrease immediately to 0·74, showing that the DPNH was completely oxidized by the enzyme, but that the irradiation product was unaffected. The final pH of the solution was found to

be 6·9, the explanation being that the enzyme was dissolved in a buffer of about this pH and the acetaldehyde had an acid reaction.

*Reduction of DPN$^+$ by substances other than ethanol*

Experiments were performed to discover whether substances other than ethanol would cause the production of an irradiation product absorbing at 340 mμ. Oxygen-free aqueous solutions of DPN$^+$ (0·27 mM) were prepared in 0·01 M sodium pyrophosphate–HCl buffer in presence of other substances at either 0·5 M or 0·01 M concentration (pH 7·8). 0·5 M solutions were irradiated with X-ray doses of 39 000 and 78 000 r, and 0·01 M solutions with doses of 39 000 r only. The absorption curve of each solution was examined for the presence of a maximum at 340 mμ. In some cases definite absorption bands appeared at 340 mμ. as with 0·5 M ethanol. In other cases it was obvious that there was no band at 340 mμ. Results are given in Table 1. In every case where an absorption band was noted at 340 mμ., air-free aqueous solutions of the substance were irradiated without DPN$^+$, to ensure that the changes were not due to the substance itself. Also a test with lactic dehydrogenase was performed. Except where the substance was lactic acid at 0·01 M concentration, it was always found that the product was not DPNH. The test was not successful with lactic acid because the enzyme was found unable to oxidize added DPNH. The reason for this is not known. None of the irradiated solutions containing the product exhibited more than a trace of visible fluorescence when examined under Wood's light. Solutions containing the substances at 0·5 M concentration were also irradiated in presence of dissolved oxygen, and under these conditions no product absorbing at 340 mμ. appeared in any case.

Table 1. *Production of a reduced form of DPN by irradiation with X-rays in presence of various substances*

| Substance | Whether an absorption band at 340 mμ. is produced by irradiating DPN$^+$ in a 0·5 M solution of the substance | Whether an absorption band at 340 mμ. is produced by irradiating DPN$^+$ in a 0·01 M solution of the substance |
|---|---|---|
| Methanol | Doubtful | Yes |
| Ethanol | Yes | Doubtful |
| *n*-Propanol | Yes | Yes |
| *iso*Propanol | Doubtful | Yes |
| *n*-Butanol | Yes | Doubtful |
| *iso*Butanol | Yes | Doubtful |
| Allyl alcohol | Yes | Yes |
| Ethylene glycol | No | Doubtful |
| Pinacol | Doubtful | No |
| Glucose | No | No |
| Lactic acid | No | Yes |
| β-Hydroxybutyric acid | Yes | Yes |
| Malic acid | No | Doubtful |
| Formic acid | Doubtful | Yes |
| Acetic acid | No | Doubtful |
| Succinic acid | No | No |
| Maleic acid | No | No |
| Benzoic acid | No | No |
| Glycine | Doubtful | Yes |

The absorption curves of DPN after irradiation in presence of 0·5 M formic acid at pH 7·8 (oxygen-free) were different in type from any of the others in that they showed an unusually large increase in optical density in the region 290–300 mμ.

*Role of hydrogen atoms in the reduction of DPN$^+$*

It was thought of interest to examine the effect of free hydrogen atoms on DPN$^+$. Experiments were performed with a palladium diffusion apparatus of the type used by Mee & Sutton in their study of the effect of hydrogen atoms on catalase (Sutton, 1952). With a current of 1 A it was first shown that 3 ml. of 0·3 mM methylene blue could be about 70% decolorized in 1 hr. It was further shown that, with a stream of air bubbling through the solution, 3 ml. of mM ferrous ammonium sulphate solution in 0·1 N-$H_2SO_4$ was 65% oxidized in 1 hr., whereas with no current flowing a control showed only 6% oxidation. It was found that the ultraviolet absorption spectra of 3 ml. portions of 0·3 mM DPN$^+$ at pH 7·8, 10·2 or 13·0 were unaffected after treatment for 1 hr. at 1 A (oxygen-free).

The yield of molecular hydrogen in oxygen-free solutions irradiated with a dose of 31 000 r has been determined. Two 100 ml. lots of 0·27 mM DPN$^+$ containing 0·5 M ethanol in 0·01 M sodium pyro-

phosphate–HCl buffer at pH 7·8 were irradiated. The amounts of gas present in the two lots were 0·18 and 0·21 ml. (corrected to s.t.p.). On combustion with 0·29 and 0·25 ml. oxygen respectively the total volumes became 0·20 and 0·22 ml. respectively. The amounts of hydrogen are given by two-thirds of the decrease in total volume on combustion, and are 0·18 and 0·16 ml., respectively. The second value is lower than the volume of gas in the irradiated solution, but the result is probably within the experimental error. The yield for the production of molecular hydrogen ($G$) is calculated from the mean of all four volumes to be 3·2 molecules produced/100 ev absorbed. In similar experiments with no $DPN^+$ present, ethanol solutions gave 0·17 and 0·21 ml. gas, and after combustion with 0·29 and 0·25 ml. oxygen the residual volumes became 0·24 and 0·22 ml. respectively. From these results $G = 2·8$. This experiment shows that the yield of hydrogen in irradiated aqueous ethanol is almost independent of the presence of $DPN^+$.

*Reaction of thiol radicals with DPN*

Theorell & Bonnichsen (1951) have discovered a compound of liver alcohol dehydrogenase with DPNH which absorbs at 325 mμ., the formation of which appears to be associated with the presence of thiol groups in the protein. They found that simple thiol compounds did not react with DPNH. Experiments were conducted to determine whether free thiol radicals, prepared by irradiating cysteine solutions (Swallow, 1952), would react with $DPN^+$ or DPNH. An oxygen-free 0·01 M sodium pyrophosphate solution was made 0·27 mM in $DPN^+$ and 0·05 M in cysteine and the pH adjusted to 7·8. It was found that no change occurred in the absorption curve on standing for about 1 hr., or on irradiation with an X-ray dose of 32 000 r. When the experiment was repeated, but with DPNH instead of $DPN^+$, it was again found that no spectral change took place on standing, and on irradiation with an X-ray dose of 32 000 r; although the absorption band at 340 mμ. decreased in intensity, there was no indication of the formation of a substance absorbing at or near 325 mμ. These results confirm Theorell & Bonnichsen's observation that cysteine does not react with $DPN^+$ or DPNH. They also indicate that under these conditions thiol radicals do not react with $DPN^+$ or DPNH to give a compound with an absorption maximum at 325 mμ.

## DISCUSSION

In a previous publication (Swallow, 1953) it was shown that irradiation with γ-rays of $DPN^+$ in oxygen-free aqueous solutions containing ethanol resulted in the production of a substance absorbing at 340 mμ., taken to be reduced DPN. The results given here show that this substance can also be produced by X-rays, and Barron, Johnson & Cobure (1954) have also reached this conclusion. It should be noted that values of the yield of irradiation product calculated by Barron *et al.* from figures published previously (Swallow, 1953) should be multiplied by four. The agreement between the yields of Barron *et al.* and those obtained by the present author is quite good.

*Nature of the irradiation product*

The lack of fluorescence of irradiated solutions, and the experiments with lactic and alcohol dehydrogenases appear to prove conclusively that the irradiation product is not identical with enzymically reduced DPN. It is therefore surprising that Barron *et al.* concluded that half the product consisted of DPNH. On the basis of the experiment with alcohol dehydrogenase reported here (which was performed after seeing the paper of Barron *et al.*) it is suggested that their experiment is capable of a different interpretation to the one given. They partly evaporated their irradiated solutions to remove acetaldehyde and some of the ethanol, and then added alcohol dehydrogenase. At $t = 0$ min., acetaldehyde was added and measurement made of the rate of decrease in optical density at 340 mμ. It is now suggested that in their experiment the removal of ethanol was not complete, and when alcohol dehydrogenase was added some of the excess of $DPN^+$ became reduced to DPNH. On adding acetaldehyde the DPNH was reoxidized, leaving only the irradiation product. If we assume the rate of evaporation to have been proportional to the vapour pressure and the mole fraction of each constituent, and that the solution was evaporated to 0·34 of the original volume, then the ethanol concentration in the solution to which enzyme was added would be 0·022 M. Taking Racker's (1950) value for the equilibrium constant of the alcohol-dehydrogenase reaction as $1·15 \times 10^{-11}$ and assuming that none of the irradiation product is DPNH, it can be shown that at $t = 0$ min. the optical density of the solution used by Barron *et al.* should have been 0·72, and that this should have been reduced to 0·41 on adding acetaldehyde. Actually, they found 0·83 reduced to 0·41, in remarkably good agreement.

Although the irradiation product is not identical with DPNH, its other properties show that it cannot be very different, and in particular that it is a reduced form of $DPN^+$. Experiments on the irradiation of $N'$-methylnicotinamide, which is a good model for DPN (Rafter & Colowick, 1954), are in agreement (Stein & Swallow, 1954). It can therefore be stated with confidence that the

irradiation product is a form of DPN reduced at the nicotinamide end of the molecule, but not in the ordinary way at C4 in the pyridine ring.

*Reaction mechanism*

It is highly probable that when water is irradiated with X- or $\gamma$-rays, the main primary act is the formation of free hydrogen atoms and free hydroxyl radicals (Weiss, 1944):

$$H_2O \rightsquigarrow H + OH.$$

In the presence of a solute capable of being oxidized in two steps, the following general type of reaction may occur (Stein & Swallow, 1954):

$$OH + AH_2 \rightarrow H_2O + AH.$$

If a second solute is present at low concentration, it will be exposed to the action of free hydrogen atoms and radicals of the type AH. Both are possible reducing agents.

Some evidence for believing that $DPN^+$ is reduced by an organic free radical rather than by hydrogen atoms has been given previously (Swallow, 1953). The results obtained with the palladium foil diffusion apparatus indicate that free hydrogen atoms, if prepared under these conditions, although capable of reducing methylene blue, cannot reduce $DPN^+$. Most conclusive, however, is the value of the yield of molecular hydrogen in irradiated aqueous solutions of ethanol. The hydrogen may derive from one or more of the following reactions:

$$2H_2O \rightsquigarrow H_2 + H_2O_2,$$
$$2H \rightarrow H_2,$$
$$H + CH_3.CH_2.OH \rightarrow CH_3.\dot{C}H.OH + H_2,$$
$$C_2H_5.OH^* \rightarrow CH_3.CHO + H_2.$$

If free hydrogen atoms were responsible for the reduction of $DPN^+$, then the introduction of $DPN^+$ into aqueous ethanol must decrease the yield of molecular hydrogen. In fact, however, the yield was found to be if anything slightly increased. It is therefore highly probable that $DPN^+$ is not reduced by hydrogen atoms, but by free hydroxyethyl radicals, as previously thought.

*Mechanism of dehydrogenase action*

From the point of view of explaining dehydrogenase action, the important feature of the present work is that $DPN^+$ has been reduced by free radicals under conditions similar to those prevailing in enzyme action, but the product is inactive. In contrast, when $DPN^+$ is reduced by sodium dithionite the product is identical with enzymically prepared DPNH, but the reaction is presumably an ionic one (Yarmolinsky & Colowick, 1954; Colowick, 1954; Swallow, 1955). It is difficult to avoid the conclusion that dehydrogenase action has more in common with the ionic reaction than with the free-radical one.

The role of the thiol group on the enzyme is of special interest with reference to alcohol dehydrogenase action. Mackinnon & Waters (1953) have suggested that the thiol group might form a free thiol radical which might initiate the reaction. However, it is not easy to see how the thiol radical itself could be formed. Also, the experiments with cysteine reported here have failed to show any interaction between cysteine and $DPN^+$ or DPNH under conditions where free radicals are present. On the other hand, the idea that the thiol group forms a hydrogen bond with the nitrogen atom of the pyridine ring (Kaplan & Ciotti, 1954; Swallow, 1954*a*) receives some experimental support from Kaplan & Ciotti's (1954) results with *Neurospora* DPNase. V. M. Clark (personal communication) has pointed out that this hypothesis also explains the fact (Theorell & Bonnichsen, 1951) that the alcohol dehydrogenase–DPNH compound absorbs at a shorter wavelength than DPNH itself. That DPNH can exist in an enzyme–substrate compound as a cationic form receives still further support from work by Rafter & Krebs (1954), who have shown that DPNH is unstable in the presence of yeast glyceraldehyde 3-phosphate dehydrogenase, and the product of breakdown has the same ultraviolet absorption spectrum as the product obtained by the addition of dilute acid.

If it is true that alcohol-dehydrogenase action proceeds through an ionic mechanism, then it would be interesting to demonstrate some form of chemical interaction between the substrate and $DPN^+$ (Burton & Kaplan, 1954). Although this has not yet been found possible with ethanol, the discovery that lactic acid appears to react chemically with $DPN^+$ (Swallow, 1955) may be equally significant.

## SUMMARY

1. Oxygen-free aqueous ethanolic solutions of $DPN^+$ give a product absorbing at 340 m$\mu$. when irradiated with X-rays.

2. The product has a similar ultraviolet absorption spectrum to DPNH, and the spectrum changes in the same way on longer irradiation or on acidification. The product, like DPNH, will reduce methylene blue.

3. The irradiation product differs from DPNH in that it does not fluoresce, and in that it does not function as a coenzyme for lactic or alcohol dehydrogenase.

4. A number of other substances can be substituted for ethanol, but no product appears when irradiated solutions contain dissolved oxygen.

5. Free hydrogen atoms appear to be unable to reduce $DPN^+$. The yield of molecular hydrogen in

ethanolic DPN solutions is the same as in ethanol solutions without DPN.

6. It has not been possible to demonstrate any interaction between free thiol radicals and $DPN^+$ or DPNH.

7. The experimental evidence supports an ionic rather than a free-radical mechanism for dehydrogenase action.

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# The Conversion of Schradan (OMPA) and Parathion into Inhibitors of Cholinesterase by Mammalian Liver

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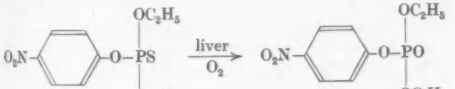
Schradan [$(Me_2N)_2PO.O.PO(NMe_2)_2$, OMPA, octamethylpyrophosphoramide, bis-*NNN'N'*-tetramethylphosphorodiamidic anhydride] and parathion (*OO*-diethyl *O*-*p*-nitrophenyl phosphorothioate) are weak inhibitors of cholinesterase *in vitro*, but when administered to animals the cholinesterase levels of the blood and tissues drop, and typical signs of acetylcholine poisoning follow (Dubois, Doull & Coon, 1950; Aldridge & Barnes, 1952; Kilby & Gardiner, 1952). It has been shown that in the presence of oxygen parathion is oxidized by mammalian liver slices to paraoxon (*OO*-diethyl *O*-*p*-nitrophenyl phosphate), a highly active inhibitor of cholinesterase (Myers, Mendel, Gersmann & Ketelaar, 1952; Gage, 1953; Metcalf & March, 1953):




Similarly, schradan is converted by mammalian liver slices in the presence of oxygen into the phosphoramide oxide and other products which are powerful inhibitors of cholinesterase (Dubois *et al.* 1950; Aldridge & Barnes, 1952; Kilby & Gardiner, 1952; Casida, Allen & Stahmann, 1954). This conversion has also been shown to occur in the liver *in vivo* (Cheng, 1951). However, the conversion does not occur in liver suspensions in the presence of oxygen. It therefore seemed interesting to investigate the difference between rat-liver slices and suspensions and to study the nature of the systems involved in the metabolism of parathion and schradan.

Since at 37° the conversion *in vitro* of both organophosphorus compounds is stopped after freezing, heating or dispersing the liver, it is concluded that enzymes probably participate in the process. It seemed possible that on dispersing the liver in buffer either an enzyme or a coenzyme was inactivated. It has now been found that the

reaction is enzymic and requires the presence of oxygen. Liver suspensions can be activated by preparing the liver in solutions containing nicotinamide with addition of a boiled liver extract or diphosphopyridine nucleotide. By differential centrifuging it has been possible to show that this oxidative conversion occurs in the liver microsomes with the addition of fortified liver supernatant. Preliminary investigations suggest that parathion and schradan are converted by similar systems in mammalian liver.

## METHODS AND MATERIALS

*Purification of parathion and schradan.* Schradan was obtained from Dr G. S. Hartley, of Pest Control Ltd., Cambridge. Traces of anti-cholinesterase impurities were removed by shaking a solution of schradan in chloroform with 1% (w/v) NaOH; after washing with water and drying, the chloroform was distilled off under reduced pressure.

Parathion was obtained from Dr H. Coates, of Albright and Wilson Ltd. The parathion was purified by shaking a chloroform solution with 1% (w/v) $Na_2CO_3$, washing, drying and distilling off the chloroform. In order to remove last traces of inhibitor a suspension of parathion (1 mg./ml.) was then prepared in 20% (w/v) gum acacia, and before use one part of this suspension was incubated for 1 hr. at 37° with an equal part of rat serum (Aldridge, 1953). The mixture was then heated to 80° for 1 min. and was then practically free from anti-cholinesterase activity.

*Pyridine nucleotides.* Diphosphopyridine nucleotide (DPN) was prepared by the method of Kornberg & Pricer (1953). The DPN preparation was found to be of 70% purity, when assayed by the methods of Gutcho & Stewart (1948) and Racker (1950). Chromatographic analysis (method of Burton & Pietro, 1954) showed that there were only traces of triphosphopyridine nucleotide (TPN) present.

*Preparation of liver suspensions and subsequent fractionation.* Fractionation of a 10% (w/v) rat-liver suspension in cold 0·25M sucrose or in cold 0·15M-NaCl, 0·015M-$MgSO_4$ and 0·008M nicotinamide was achieved at 0° by the differential centrifuging technique of Schneider (1948). Microsomes were separated from the soluble clear fraction of liver by centrifuging at 18 000*g* for 1 hr. at 0°. The material for an active preparation was prepared for routine use by centrifuging a cold suspension of rat liver (25%, w/v) in NaCl, $MgSO_4$ and nicotinamide solution at the maximum speed in a Minor (MSE) centrifuge for 10 min. at room temp.

*The formation and estimation of the anti-cholinesterase inhibitors from schradan and parathion.* Since the amounts of the organophosphorus compounds that are oxidized are very small, it proved impossible to measure the oxygen taken up in the conversion process. Inhibition of horse-serum pseudocholinesterase has therefore been used throughout this work.

The conversion of organophosphorus compounds was effected in air at 37° in Warburg flasks. Cofactors and inhibitors were added to 2 ml. of a liver preparation in the centre well of a flask and the organophosphorus compound was placed in the side arm. After 5 min. shaking at 37°, the contents of the side arm were tipped into the well of the flask. Controls were run: (1) by adding the organophosphate from the side arm at the end of the experiment, (2) by boiling the liver preparation or (3) by gassing with $CO_2 + N_2$ (5:95) in place of air. The contents of the flasks were suitably diluted with buffer and 0·5 ml. added to 1 ml. of horse serum (diluted 15-fold with buffer) in a Warburg flask together with 2 ml. of bicarbonate buffer (0·0357M-$NaHCO_3$, 0·04M-$MgCl_2$ and 0·164M-NaCl). After gassing with $CO_2 + N_2$ (5:95) and incubating at 37° for 30 min. the cholinesterase activity was determined manometrically by addition of butyrylcholine (Davison, 1953) and the activity was expressed as a percentage of control. The various inhibitors and cofactors added to the liver preparations were found not to interfere with this estimation. The units of inhibitor formed were expressed as 10 (2 − log% activity)/10 mg. of protein (determined by the biuret method), 10 units being equivalent to approximately $3 \times 10^{-6}$ mole metabolite from schradan/10 mg. of protein (Casida, Chapman, Stahmann & Allen, 1952) and to $6 \times 10^{-8}$ mole paraoxon/10 mg. of protein.

## RESULTS

### *The failure of rat-liver suspensions to convert schradan*

In agreement with others we have found that rat-liver suspensions oxygenated in Krebs–Ringer phosphate, in saline or bicarbonate–saline do not convert added schradan, although intact cell preparations will do so readily (see Table 2). Fleisher & Jandorff (1952) have claimed that this is due to the destruction of schradan by the liver suspension. However, if the supernatant obtained by centri-

Table 1. *The stability of schradan in liver suspensions*

Cooled rat-liver slices (2 g.) were put into a Warburg flask (1) containing 4 ml. of 0·0357M-$NaHCO_3$, 0·164M-NaCl buffer; 6 ml. of a 33·3% (w/v) rat-liver suspension in the same buffer was pipetted into a second flask (2). Schradan was added to each flask to give a final concentration of 0·5 mg./ml. After shaking at 37° in air for 30 min., the flask contents were centrifuged down; 0·5 ml. of the supernatant of each was incubated for 30 min. with horse serum (diluted 15-fold) and the inhibition of cholinesterase determined. Liver (1 g.) was then suspended in 3 ml. of supernatant from flask (1) and 1 g. of liver slices added to 3 ml. of supernatant from flask (2). The two preparations were placed in Warburg flasks (3 and 4) and shaken in air at 37° for 30 min. The anti-cholinesterase activity was again determined. Chloroform-soluble organic phosphate was also determined (method of Aldridge, 1954).

| Flask no. | Preparation | Inhibition of cholinesterase (%) | Chloroform-soluble phosphorus (μg./ml.) |
|---|---|---|---|
| 1 | Slices + schradan | 100 | 54 |
| 2 | Suspension + schradan | 0 | 60 |
| 3 | (1) + suspension | 100 | — |
| 4 | (2) + slices | 100 | — |
| 5 | Schradan alone | 0 | 60 |

fuging a liver suspension, previously incubated for 30 min. in air with schradan, is added to fresh rat-liver slices an inhibitor is readily formed (Table 1). Furthermore, the anti-cholinesterase produced by incubation of liver slices and schradan is itself not destroyed by liver suspensions. It therefore seems probable that the results obtained by Fleisher & Jandorff are due to the presence of impurities in technical schradan, which are in fact destroyed by incubating with liver suspensions (see also Casida & Stahmann, 1953).

*The activation of liver suspensions by cofactors*

The failure of liver suspensions to convert schradan would therefore appear to be due either to lability of an enzyme system or to dilution or destruction of cofactors normally present in intact cells. Since the addition of boiled yeast or liver extracts to liver suspensions resulted in some conversion being obtained, it seemed probable that on dispersion of the liver an essential cofactor was lost.

Addition of adenine nucleotides did not enhance conversion, but if nicotinamide and diphosphopyridine nucleotide (DPN) were added, schradan was effectively converted into its active metabolite (Table 2). A boiled liver suspension plus DPN did not oxidize schradan. It was also found that the addition of $Mg^{2+}$, and to a lesser extent $Co^{2+}$, increased the ability of liver suspensions to produce the anti-cholinesterase. Addition of $Fe^{3+}$, $Mn^{2+}$, $Ca^{2+}$, $K^+$ or $PO_4^{3-}$ had no such effect. Since it was found that addition of $Mg^{2+}$ to liver suspensions did not affect the stability of any anti-cholinesterase formed from schradan, it seems that $Mg^{2+}$ is a participant in the enzyme system. A further improvement in the metabolism of schradan could be obtained by centrifuging suspensions for 10 min. at 8500 *g* (Table 2). This improvement is possibly due to the separation of a nucleotidase in the whole liver suspension (see Carruthers & Suntzeff, 1954). Experiments on the stability and rates of reversal of inhibited cholinesterase, using the metabolites from slices and suspensions, indicate that the same compound is produced by each preparation (Table 3).

Table 2. *The conversion of schradan by liver preparations*

Different liver preparations were shaken in air with schradan (0·5 mg./ml.) at 37°. The amount of inhibitor produced was determined on the supernatant after centrifuging by the method described in the text.

| Liver preparation | Inhibitor units/10 mg. protein produced in 30 min. at 37° |
|---|---|
| 1. Rat-liver slices (1 g.) in 4 ml. of saline mixture | 12 |
| 2. Rat-liver (1 g.) squeezed through a glass syringe in 4 ml. of saline | 25 |
| 3. Liver suspension (25%, w/v) in Krebs–Ringer phosphate | 0·01 |
| 4. As (3) + 0·008 M nicotinamide | 6·3 |
| 5. Liver suspension (25%, w/v) in 0·15 M-NaCl, 0·015 M-$MgSO_4$ + 0·008 M nicotinamide | 9·5 |
| 6. As (5) + DPN (0·4 mg./ml.) after suspending | 30 |
| 7. As (6) but centrifuged 10 min. at 8500 *g* with DPN (0·4 mg./ml.) added afterwards | 48 |

Table 3. *Constants for the metabolites of schradan*

Schradan (0·5 mg./ml.) was added to rat-liver slices (1 g.) in 4 ml. of 0·15 M-NaCl, 0·015 M-$MgSO_4$ solution or to a rat-liver suspension (25%, w/v) in the same solution together with 0·008 M nicotinamide and DPN (0·4 mg./ml.). After shaking in air for 30 min. at 37°, the metabolite formed was extracted by shaking 4 ml. of the centrifuged supernatant with 4 ml. of chloroform. The chloroform layer was separated and the chloroform distilled off under reduced pressure; the residue was dissolved in 10 ml. of Sørensen's 0·067 M phosphate buffer (pH 7·6). Buffer solutions containing metabolite from slices or suspensions were kept at 37°, and the rate of loss of anti-cholinesterase activity determined at intervals by incubating 0·5 ml. with horse serum for 30 min. as described in the text. The rate of reversal of cholinesterase inhibition was determined by the method of Davison (1953) after inhibition of 4·5 ml. of rat serum by 0·5 ml. of the supernatant from slices or suspension.

| | Slices | Suspension |
|---|---|---|
| Rate of hydrolysis of schradan metabolite | $3 \times 10^{-2}$ min.$^{-1}$ | $3 \times 10^{-2}$ min.$^{-1}$ |
| Half-life for reversal of inhibition by the schradan metabolite | 28 hr. | 28 hr. |

*Distribution of the enzyme system converting parathion and schradan*

Separation of rat-liver suspensions in 0·25 M sucrose or in the NaCl–nicotinamide–$MgSO_4$ solution was effected by differential centrifuging. It was found that neither the cells and nuclei nor the mitochondria converted either schradan or parathion, although the mitochondria could oxidize added glutamate, choline or tyramine. The major part of the activity remained in the supernatant after removal of the mitochondria. This fraction was further separated into microsomes and a clear supernatant, but neither fraction was as efficient alone as the two fractions together (Table 4). It is interesting to note that the enzyme converting parathion and schradan shows a similar

distribution. It has also been found that tri-*o*-cresyl phosphate (TOCP) (Aldridge, 1954) and *OO*-dimethyl *O*-*p*-nitrophenyl phosphorothioate are converted by fortified liver suspensions from weak inhibitors into active anti-cholinesterases.

Table 4. *The distribution of the converting enzyme in rat-liver fractions*

Liver suspensions (10%, w/v) in 0·15M-NaCl, 0·015M-$MgSO_4$ and 0·008M nicotinamide were separated by differential centrifuging at 0° (Schneider, 1948). DPN (0·4 mg./ml.) was added to each fraction together with schradan (1 mg./ml.) or parathion (200 $\mu$g./ml.) in suspension. After 30 min. shaking in Warburg flasks in air at 37°, the anti-cholinesterase activity of suitable dilutions of the different preparations was determined as described in the text. The mitochondria used were shown to be active against glutamate, choline and tyramine.

| Liver fractions | Units of inhibitor produced by: Schradan | Parathion |
|---|---|---|
| Cells and nuclei | 0 | — |
| Mitochondria | 0 | — |
| Microsomes | 2 | 160 |
| Clear supernatant | 17 | 130 |
| Microsomes + clear supernatant | 50 | 910 |

Table 5. *Conversion of schradan and parathion by male and female rat liver*

Chilled liver was squeezed through a glass syringe into the nicotinamide–NaCl–$MgSO_4$ solution (25%, w/v). Schradan (1 mg./ml.) or parathion (200 $\mu$g./ml.) was added. The organophosphate and liver brei were shaken in air at 37° for 30 min. and the number of units of inhibitor produced determined. Values for $LD_{50}$ for parathion are those of Dubois *et al.* (1949) and for schradan are those observed in this laboratory.

| | Males | Females |
|---|---|---|
| $LD_{50}$ of schradan (mg./kg.) | 7 | 27 |
| Units obtained *in vitro* from schradan | 35, 40, 40 | 15, 16, 20, 20 |
| $LD_{50}$ of parathion (mg./kg.) | 7 | 4 |
| Units obtained *in vitro* from parathion | 7·5, 7·5 | 69, 48 |

*The distribution of the converting system in rat tissues and the effect of sex differences*

Liver, brain, kidney or spleen was forced through an all-glass syringe (Aldridge, personal communication) to give a tissue brei in the saline mixture. Only the liver brei converted added schradan into an active anti-cholinesterase.

Since schradan is more toxic to male rats and parathion more toxic to female rats, it seemed interesting to see if the tissue brei preparation of the liver of male and female rats differed in converting ability. It was found that the liver preparation of male rats was about twice as efficient as the female in converting schradan (Table 5). Female rat livers were much more efficient than male in converting parathion.

*Requirement for added DPN*

It has been shown that addition of nicotinamide is necessary for the conversion of schradan and parathion by rat-liver suspensions. This conversion is considerably enhanced if DPN is added (Table 2). The rate of production of an anti-cholinesterase from schradan by rat-liver microsomes and supernatant with added DPN was proportional to time (Fig. 1), but, if a brain suspension containing a

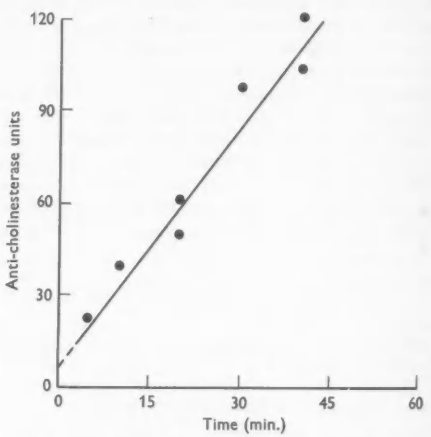



Fig. 1. The rate of production of inhibitor from schradan by rat liver. An active preparation of rat-liver microsomes was made as described in the text. Schradan (1 mg./ml.) and DPN (0·4 mg./ml.) were added and the whole was shaken in air at 37° for varying times.

pyridine nucleotidase was added, the rate of conversion was considerably reduced. Addition of increasing amounts of three different preparations of DPN to the microsome preparation (Fig. 2) resulted in increased production of anti-cholinesterase, although addition of more than $1 \times 10^{-3}$M DPN resulted in less than the optimum production of inhibitor. Reduced DPN was about 90% as efficient in converting as DPN when added to the microsome-supernatant preparation plus schradan. In another series of experiments reduced DPN (Gutcho & Stewart, 1948) was added to the liver microsome-supernatant preparation. The oxidation of DPNH at 37° was followed by the decreased absorption at 340 m$\mu$. in a Unicam spectrophotometer (SP. 500). Addition of schradan decreased the rate of oxidation. This suggests that some DPN

is reduced by the system when schradan is added and that oxidized DPN is therefore necessary for the system.

Results previously obtained suggest that TPN or TPNH cannot effectively replace DPN in the enzyme system converting schradan or parathion (Davison, 1954). Furthermore, it has so far been impossible to demonstrate that microsomes by themselves or with addition of oxidized or reduced pyridine nucleotide convert either schradan or parathion. Addition of DPN to liver suspensions without added nicotinamide resulted in a preparation about one-third as active as if nicotinamide had been present; however, the absence of nicotinamide in the microsome-supernatant preparation resulted in only slight loss of activity.

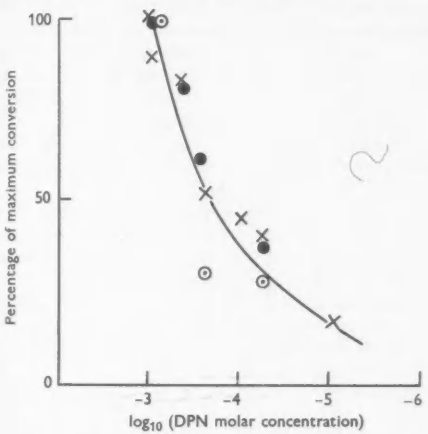



Fig. 2. The effect of addition of DPN on the conversion of schradan. Nicotinamide was added after centrifuging a rat-liver suspension (25%, w/v) in NaCl–$MgSO_4$ at 8500 *g* for 10 min. at room temp. Then DPN at varying concentrations and schradan (1 mg./ml.) were added to different flasks and the production of anti-cholinesterase on shaking in air for 30 min. at 37° was determined. Our own DPN (×), DPN from Light and Co. Ltd. (⊙) and from Boehringer and Soehne, Germany (●) were used.

### *Properties of the active enzyme system*

The fortified liver microsome preparation was highly thermolabile. Thus, warming to 50° for 10 min. destroyed all its activity. The optimum pH for the conversion of both parathion and schradan is near neutrality (Fig. 3). The action of a number of different types of inhibitor on the conversion process has also been studied. It will be seen that the oxidation of the two organophosphorus compounds by rat-liver microsome preparations shows a similar sensitivity to inhibition (Table 6). The

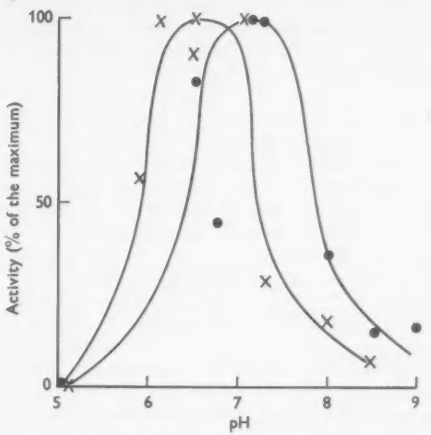



Fig. 3. The pH optimum curves for conversion of schradan and parathion. Equal volumes of the fortified microsome-supernatant preparation and either Sørenson's phosphate or McIlvaine's phosphate–citric acid buffers were added together with the organophosphate and the pH was determined. After shaking in air for 30 min. at 37° the anti-cholinesterase produced was measured. Concentrations of 1 mg./ml. schradan (●), and 200 μg./ml. parathion (×) were used.

Table 6. *The inhibition of the conversion of schradan and parathion*

An active microsome supernatant preparation was obtained by suspending chilled rat liver (25%, w/v) in 0·15 M-NaCl, 0·015 M-$MgSO_4$ and 0·008 M nicotinamide and centrifuging for 10 min. at 8500 *g* at room temp. DPN (0·4 mg./ml.) and 1 mg./ml. schradan or 200 μg./ml. parathion in suspension were added. The inhibitors were added to the conversion mixture 5 min. before addition of the organophosphorus compound. Percentage inhibition was calculated as the inhibition of anti-cholinesterase produced in 30 min. at 37° compared to a control without inhibitor.

| Inhibitor added | Final concentration (M) | Percentage inhibition of the conversion of | |
|---|---|---|---|
| | | Schradan | Parathion |
| Mercuric chloride | $10^{-3}$ | 100 | 90 |
| Iodoacetate | $10^{-3}$ | 84 | 100 |
| *p*-Chloromercuribenzoate | $10^{-4}$ | 33 | 66 |
| Chloropicrin | $10^{-3}$ | 100 | 100 |
| Hydroxylamine | $10^{-3}$ | 100 | 90 |
| 2:4-Dinitrophenol | $10^{-3}$ | 100 | 100 |
| Potassium cyanide | $10^{-4}$ | 15 | 0 |
| Potassium cyanide | $10^{-5}$ | 0 | 0 |
| Choline | $10^{-3}$ | 52 | 93 |
| Trimethylamine | $10^{-3}$ | 39 | — |
| Dimethylamine | $10^{-3}$ | 38 | — |
| SKF 525A | $10^{-3}$ | 54 | 52 |

compound SKF 525A [2-(diethylamino)ethyl diphenylpropylacetate hydrochloride] also inhibits both conversions. This seemed particularly interesting, as La Du, Trousof & Brodie (1952) have shown that this substance inhibits the de-alkylation of aminopyrine and other alkylamines *in vitro* by a liver microsome preparation and *in vivo*. In view of these results the effect of SKF 525A has been examined *in vivo*. SKF 525A (100 mg./kg.) was given by intraperitoneal injection to six male rats of about 250 g. After 20 min. these animals, and a further six untreated rats, were given 8 mg./kg. of schradan by intraperitoneal injection. Within 40 min. all six rats of the control group had died with typical signs of acetylcholine poisoning and only one of the SKF group (this animal did not show signs of acetylcholine poisoning). The remaining rats survived with no signs of poisoning. Similar experiments with parathion (10 mg./kg.) resulted in signs of acetylcholine poisoning (red tears, etc.) lasting for 24 hr. in the pretreated group, although in this case four out of the SKF group died compared to three from the control group of six.

## DISCUSSION

It is now recognized that the liver is the site for the metabolism of a number of *N*-dimethyl compounds (Butler & Waddell, 1954). It is interesting therefore that not only is schradan metabolized by mammalian liver but also that entirely different compounds, parathion, tri-*o*-cresyl phosphate and *OO*-dimethyl *O*-*p*-nitrophenyl phosphorothioate, are oxidized by the same tissue. Several workers have shown that rat, rabbit or mouse-liver slices convert either schradan or parathion into powerful inhibitors of cholinesterase and that liver suspensions failed to convert either compound. Addition of nicotinamide and diphosphopyridine nucleotide has now been found to be necessary for the optimum production of an anti-cholinesterase from schradan by whole rat-liver suspensions.

Using the differential centrifuging technique of Schneider (1948), it has been found that conversion of both schradan and parathion occurs in the microsome plus supernatant fraction of the liver and DPN (Davison, 1954). Omission of nicotinamide in this preparation resulted in slight loss of activity.

Brodie and his associates have found that barbiturates, sympathomimetic amines and thiopentonal are all oxidized by liver microsomes plus the supernatant, $Mg^{2+}$ and TPN (Brodie & Cooper, 1954; Axelrod, 1954). The supernatant and TPN can be replaced by reduced TPN or any system that will generate TPNH. Since there are many similarities between the preparation of Brodie and our own, it seemed interesting to see if DPN could be replaced by TPN or TPNH. The results (Davison, 1954) suggest that TPN or TPNH will not replace DPN. However, this conclusion can only be provisional in that the sample of TPN used in those studies was grossly impure. Injection of the compound SKF 525A increases the survival time and prolongs the signs of acetylcholine poisoning in rats treated with schradan and possibly parathion; furthermore, *in vitro* SKF 525A inhibits the conversion process. It is interesting that SKF 525A also inhibits the oxidation of barbiturates both *in vivo* and *in vitro* (Kensler, Matchett & Bradley, 1954), while iodoacetate has been shown to inhibit the oxidation of barbiturates *in vitro* (Brodie & Cooper, 1954).

Mercuric chloride, iodoacetate, *p*-chloromercuribenzoate and chloropicrin inhibit the conversion of parathion and schradan. This suggests that SH groups are an essential part of the system. The absence of any inhibitory effect by cyanide shows that cytochrome oxidase does not participate in the reaction; it is therefore interesting to note that DPN–cytochrome *c*–reductase found chiefly in the liver microsomes (Brody, Bain & Wang, 1952) is sensitive to inhibition by *p*-chloromercuribenzoate but not by cyanide (Mahler & Elowe, 1954). Choline, trimethylamine, dimethylamine and SKF 525A also inhibit the conversion, possibly by competitive inhibition. The enzyme systems for conversion of schradan and parathion are sensitive to the same temperature changes and both have about the same optimum pH. These results suggest that the oxidation of schradan and parathion is effected by similar enzyme systems.

Despite these similarities at least one basic difference between the two systems has been found. The liver of male rats converts schradan better than does the liver of female rats and the reverse holds for parathion. The active metabolite from schradan is not very rapidly hydrolysed *in vitro* by liver suspensions and there is no evidence to suggest that the cholinesterase of female rats is more susceptible than that of males to inhibition by organophosphorus compounds. Thus male and female rats are equally sensitive to poisoning by two compounds which do not undergo conversion *in vivo*, tetraethyl pyrophosphate and *OO*-diethyl *O*-*p*-nitrophenyl phosphate (Aldridge & Barnes, 1952). This sex difference in the metabolism of the compounds, also seen in the oxidation of barbiturates (Quinn, Axelrod & Brodie, 1954), may account for the fact that schradan is more toxic to male rats than to female (Aldridge & Barnes, 1952), while parathion is more toxic to female rats than to male (Dubois, Doull, Salerno & Coon, 1949). In spite of these differences it can be concluded that the enzyme system responsible for the metabolism of schradan, parathion and probably

tri-*o*-cresyl phosphate is located in liver microsomes plus the supernatant and is active with the addition of nicotinamide, $Mg^{2+}$ and diphosphopyridine nucleotide. This metabolic system probably has a very broad specificity, for not only are barbiturates and amines (Brodie & Cooper, 1954; Axelrod, 1954) oxidized by a similar system, but also 4-dimethylaminoazobenzene (Mueller & Miller, 1948) and 4-aminoimidazole-5-carboxamide [4(5)-amino-5(4)-carbamoylglyoxaline] (Miller & Warren, 1953) are oxidized by rat-liver suspensions only after addition of nicotinamide, $Mg^{2+}$ and diphosphopyridine nucleotide. Furthermore, the enzyme system of insects which converts added parathion and schradan into anti-cholinesterase resembles the mammalian system in all respects so far investigated (Metcalf & March, 1953; O'Brien & Spencer, 1953; Kok & Walop, 1954). All these findings suggest that the microsome–supernatant system studied in this paper has an important role in fundamental metabolism.

## SUMMARY

1. Parathion, schradan, tri-*o*-cresyl phosphate and *OO*-dimethyl *O*-*p*-nitrophenyl phosphorothioate have been shown to be oxidized *in vitro* by a rat-liver preparation to powerful inhibitors of cholinesterase.

2. The enzyme system is located in the microsome-plus-supernatant fraction of liver suspensions.

3. Diphosphopyridine nucleotide and magnesium ions are necessary for the conversion process *in vitro*.

4. Cytochrome oxidase is not an essential participant in the reaction, but it has been shown that the system is sensitive to thiol group inhibitors.

5. The conversion of parathion and schradan is mediated by similar but not necessarily identical systems.

My thanks are due to Dr W. N. Aldridge for his interest and criticism, and to Miss J. I. Wheatley for technical assistance. Dr K. Carter of Smith, Klyne and French Ltd. kindly supplied the SKF 525A.

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# Ammonia Formation in Brain

## 1. STUDIES ON SLICES AND SUSPENSIONS

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It has been known for some time that brain tissue is capable of forming considerable amounts of ammonium ions *in vivo* and *in vitro* (for review of literature see Weil-Malherbe, 1950, 1953*b*), but its origin and the mechanism of its formation are still obscure. The experiments reported in this paper were undertaken to study these problems further.

## METHODS

### *Tissue preparations and conditions of incubation*

Slices of brain grey matter were cut freehand with a moist single-edged safety-razor blade and stored in ice-cold bicarbonate–Ringer solution. Batches of 60–80 mg. (wet wt.) were used in 3 ml. suspension medium. A similar batch was used for the estimation of the wet wt./dry wt. ratio. Wet weights were corrected for swelling by multiplying the calculated dry wt. with an assumed wet wt./dry wt. ratio of 5·0.

Whole rat brains were used for the preparation of suspensions. The tissue was ground in a Potter–Elvehjem homogenizer in 1 vol. of medium and diluted to the required strength.

Brain slices were suspended in bicarbonate–Ringer solution (Krebs & Henseleit, 1932). The following media were used in experiments with brain suspensions: (1) a solution of 0·020M-$NaHCO_3$ ('water suspension'); (2) an alkaline potassium-rich salt solution (modified from Potter, 1948), containing KCl 0·05M, $KHCO_3$ 0·05M, potassium phosphate buffer (pH 7·6) 0·01M; and (3) an isotonic saline (modified from Birmingham & Elliott, 1951), containing NaCl 0·12M, $NaHCO_3$ 0·025M, KCl 0·003M, $KH_2PO_4$ 0·0004M, $MgSO_4$ 0·0012M. The final concentration of the brain suspensions was 1 in 30.

In some experiments brain suspensions were used after dialysis against running isotonic salt medium for 3 hr. at 3° or after three washings of the centrifuged residue with the same medium.

Rat-brain mitochondria were collected as the fraction thrown down between 1200 and 15000 *g* in isotonic sucrose. The residue was resuspended 3 times in sucrose and incubated in a medium containing KCl 0·1M, $MgSO_4$ 0·01M, and potassium phosphate buffer (pH 7·4) 0·05M.

Unless specifically denoted as anaerobic, all incubations were carried out in an atmosphere of 5% (v/v) $CO_2$ in $O_2$. A gas mixture of 5% (v/v) $CO_2$ in $N_2$, freed from oxygen by passage over red-hot copper wire, was used in anaerobic experiments. The flasks were shaken in a water bath at 37°.

### *Analytical*

*General.* In experiments with brain slices 0·1 vol. 50% (w/v) trichloroacetic acid (TCA) was added at the end of the incubation period or, where initial values were required, before the addition of the slices. The tissue was thoroughly broken up with a glass rod and the mixture filtered through Whatman no. 5 paper. The whole of the filtrate or a suitable portion was used for the estimation of free ammonia and amide N. When other fractions were to be determined, the mixture of slices, medium and TCA was rinsed into a graduated centrifuge tube with 5% (w/v) TCA, some acid-washed sand added and, after thorough grinding, the volume made up to 6 ml. with 5% TCA. After quickly centrifuging, the supernatant was filtered through Whatman no. 5 paper. Portions of 0·5–1 ml. of filtrate were used for the estimations of acid-soluble P and non-protein N (NPN) and the remainder was used for the estimation of free ammonia and amide N.

In experiments with brain suspensions, 2-ml. samples were withdrawn with a wide-tipped pipette and delivered into 2 ml. 12% (w/v) TCA. When initial values were to be determined, this was done immediately after the addition of the brain suspension. Of the filtrate, 2 ml. were used for the estimation of free ammonia and amide N and 1 ml. for the estimation of NPN.

*Estimation of ammonia and amide N.* These two estimations were carried out in the same solution by the method of Krebs (1935). A distillate of 10 ml. was collected in the apparatus of Parnas & Heller (1924). When only free ammonia was estimated, Conway units were used with 2 ml. filtrate and 1 ml. saturated $K_2CO_3$ in the outer ring (cf. Conway, 1950), followed by nesslerization of the solution contained in the centre compartment (see below).

*Estimation of total nitrogen.* Micro-Kjeldahl determinations were carried out by the Conway microdiffusion method after digestion with a mercury catalyst. Since the use of a mercury catalyst has been considered inconvenient or unsuitable in conjunction with the microdiffusion technique (Conway, 1950), particulars of the procedure are given. To the sample containing 5–50 $\mu$g. N is added 1 ml. of a solution containing 0·625% (w/v) HgO in 9N-$H_2SO_4$. After the water has been boiled off, 100 mg. $K_2SO_4$ are added. Digestion is continued for 2 hr. after clearing. The cooled digestion mixture is diluted to 5 ml. and a sample of 2 ml. is placed in the outer chamber of a Conway unit, followed by 1 ml. of a solution containing 10% (w/v) $Na_2S_2O_3,5H_2O$ in 60% (w/v) KOH. The inner chamber contains 1 ml. 0·01N-HCl. When the diffusion of ammonia is complete the contents of the inner chamber are removed with a fine-tipped teat pipette and transferred to a test

tube, followed by two washings with 1 and 0·5 ml. water respectively; 0·25 ml. Nessler reagent is then added. Readings are taken in colorimeter cells with capacity 3·5 ml. and diam. 1 cm.

The method gave theoretical recoveries with various purines and amino acids.

*Phosphate fractions.* After a further washing in 5% TCA the insoluble tissue residue was fractionated by the method of Schmidt & Thannhauser (1945) as modified by LePage & Heidelberger (1951). The total N was estimated in a sample of the alkaline digest and used for the calculation of 'protein N' as described by Leslie & Davidson (1951). Total P (5–10 $\mu$g./sample), after digestion with 60% (w/w) perchloric acid (King, 1932), was estimated as previously described (Weil-Malherbe & Green, 1951; Weil-Malherbe, 1953*c*).

### *Materials*

*Diphosphopyridine nucleotide.* This was prepared according to LePage (1947). The substance was standardized spectrophotometrically according to Kornberg (1950); it was 48·5% pure.

*Inosine triphosphate* (ITP). This was prepared according to Lehninger (1945). The Ba salt had: inorganic P, 0·16; organic P, 10·3; acid-labile P, 6·61; N, 6·44%. Calc. for $C_{10}H_{11}O_{14}N_4P_3Ba_2,7H_2O$: organic P, 10·3; acid-labile P, 6·86; N, 6·19%.

*Creatine phosphate.* This was prepared according to Zeile & Fawaz (1938) and converted into the barium salt. (Found: inorganic P, 0·29; organic P, 8·76; acid-labile P, 8·78%. Calc. for $C_4H_9O_5N_3PBa$: P 8·95%.)

D-*Glutamic acid.* This was a gift from Professor H. A. Krebs. For analytical data see Krebs (1935).

*Cytochrome* c. Landrax (Evans Medical Supplies Ltd., Liverpool and London) was dialysed until free of ammonium ion.

*Other materials.* These were of commercial origin and were used without further purification.

## RESULTS

### *Experiments with brain slices*

*Ammonia formation.* It has previously been shown (Weil-Malherbe, 1936) that brain slices respiring in a glucose-free medium form ammonia at a fairly steady rate for several hours. This observation has now been confirmed by a larger number of experiments (Fig. 1). In most experiments slices of guinea-pig brain were used, but very similar results were also obtained with slices of rat brain. The curve of ammonia formation is slightly S-shaped with its steepest part in the period 0·5–2 hr.

The rate of ammonia formation is strongly and progressively inhibited under anaerobic conditions as well as aerobically in the presence of mM 2:4-dinitrophenol (DNP). In Ringer solution containing 0·2% glucose the ammonia formation is completely suppressed during the first 2 hr. and slightly increases thereafter. The inhibition by glucose and other substrates of the ammonia formation by brain slices has previously been observed by Warburg, Posener & Negelein (1924), Loebel (1925), Dickens & Greville (1933) and Weil-Malherbe (1936).

*Amide nitrogen.* When guinea-pig brain slices are respiring in a substrate-free medium, the amide concentration decreases during the first hour (Fig. 2). This change is probably significant ($P = 0·04$), but the subsequent increase found after 2 hr. is not ($P = 0·09$); neither is the difference between the initial and the final values ($P = 0·16$). Under these conditions, therefore, the amide concentration appears to remain more or less constant

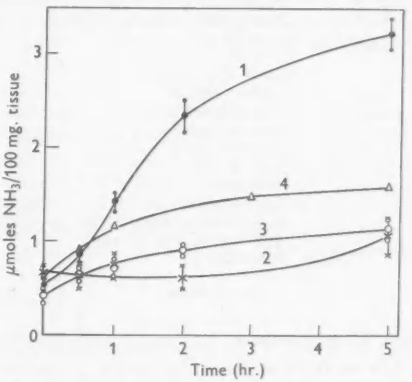


Fig. 1. Ammonia formation of guinea-pig brain slices. Curve 1 (●—●), in glucose-free Ringer soln. (means of 13 expts. with S.E.); curve 2 (×—×), in glucose–Ringer soln. (means of 6 expts. with S.E.); curve 3 (○—○), in glucose-free Ringer soln., anaerobic (means of 4 expts. with S.E.); curve 4 (△—△), in Ringer soln. containing $10^{-3}$M 2:4-dinitrophenol.

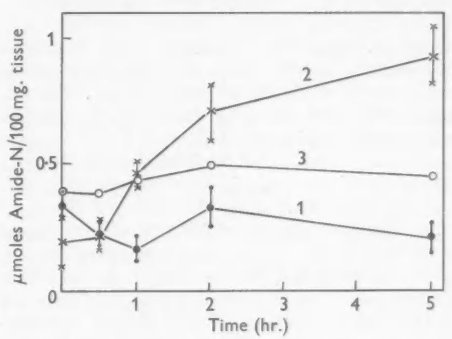


Fig. 2. Concentration of amide N in guinea-pig brain slices. Curve 1 (●—●), in glucose-free Ringer soln. (means of 9 expts. with S.E.); curve 2 (×—×), in glucose–Ringer soln. (means of 5 expts. with S.E.); curve 3 (○—○), in glucose-free Ringer soln., anaerobic (means of 3 expts.).

for several hours. It is also stable under anaerobic conditions. In the presence of glucose, on the other hand, a steady and significant rise of the amide concentration is found in aerobic experiments.

*Effect of inhibitors on ammonia formation.* In addition to DNP, the effect of other inhibitors on the ammonia formation of brain slices was studied (Table 1). Significant inhibitions were produced by those that are known to interfere with electron transport or oxidative phosphorylation. On the other hand, some that might have been expected to influence ammonia formation were found to have no effect. Fluoroacetate, for instance, increases the formation of ammonia in brain *in vivo* (Benitez, Pscheidt & Stone, 1954), but does not do so *in vitro*. Ephedrine is an inhibitor of amine oxidase (Blaschko, Richter & Schlossmann, 1937; Mann & Quastel, 1940), but does not affect the ammonia formation of brain slices. The same is true for methionine sulphoxide, which inhibits the synthesis of glutamine (Borek, Miller, Sheiness & Waelsch, 1946). D-Glutamic acid is a potent inhibitor of glutaminase (Krebs, 1935) and virtually abolishes the ammonia formation from added glutamine by brain slices, yet it does not inhibit the ammonia formation occurring without added substrate (Table 2).

Sulphanilamide caused a slight increase of ammonia formation, which is probably significant since it was observed on three occasions. The possibility that sulphanilamide was itself deaminated cannot be excluded, however.

Table 1. *Ammonia formation of guinea-pig brain slices in the presence of various substances*

| Addition | Incubation, 4 hr. Concentration (M) | No. of expts. | Change or mean change (%) |
|---|---|---|---|
| Hydrazine | $10^{-2}$ | 3 | −67·4 |
| Octan-2-ol | Saturated soln. | 3 | −66·0 |
| Arsenite | $10^{-3}$ | 3 | −57·3 |
| Cyanide | $10^{-3}$ | 1 | −42·5 |
| Iodoacetate | $10^{-3}$ | 1 | −42·4 |
| Hydroxylamine | $10^{-3}$ | 1 | −42·0 |
| Sodium fluoride | $10^{-2}$ | 1 | −38·3 |
| Sodium sulphide | $10^{-3}$ | 1 | −35·0 |
| Methylene blue | 0·05% (w/v) | 1 | −28·0 |
| Sodium azide | $10^{-3}$ | 1 | −26·6 |
| Urethane | $10^{-2}$ | 3 | −25·5 |
| Fluoroacetate | $0·5 \times 10^{-3}$ | 3 | − 8·6 |
| DL-Methionine sulphoxides (mixture of four isomers) | $10^{-2}$ | 2 | − 5·5 |
| Ephedrine | $10^{-2}$ | Time curve with 5 points | ± 0 |
| Sulphanilamide | $0·5 \times 10^{-3}$ | 3 | +24·1 |

Table 2. *Effect of glutamine and* D-*glutamic acid (both at* 0·002 M*) on the ammonia formation of guinea-pig brain slices*

| Addition | Incubation, 3 hr. $NH_3$ formed (μmoles/100 mg. tissue) |
|---|---|
| None | 1·62 |
| L-Glutamine | 6·65 |
| D-Glutamic acid | 1·65 |
| L-Glutamine + D-glutamic acid | 2·07 |

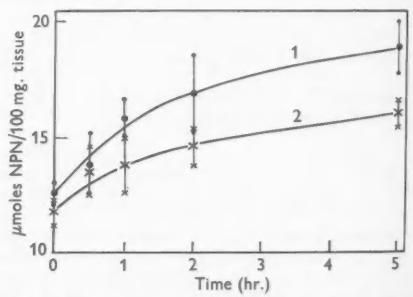


Fig. 3. Concentration of non-protein N in guinea-pig brain slices. Curve 1 (●—●), in glucose-free Ringer soln. (initial and final values are the means of 16 expts., intermediate values the means of 6 expts., with S.E.); curve 2 (×—×), in glucose–Ringer soln. (initial and final values are the means of 13 expts., intermediate values the means of 6 expts., with S.E.).

In addition to those mentioned in Table 1 the following substances were tested, but they had no significant effect on the ammonia formation of brain slices: arsenate, oxalate, malonate, maleate, malononitrile, acetonitrile, benzaldehyde (all at 0·01 M); creatine phosphate, ITP, inosinic acid (at 0·0025 M); Congo red, pyocyanine, porphyrindin (at 0·05%); *p*-chloromercuribenzoate, benzylpenicillin, *o*-phenanthroline (at 0·0005 M).

*Non-protein nitrogen.* As shown in Fig. 3 there is a significant increase of NPN during a 5 hr. incubation of brain slices, both in the presence and in the absence of glucose ($P<0.01$), but the effect is greater in the glucose-free medium. The difference between the two media is probably significant after 5 hr. ($P=0.05$), though not for the earlier values, but this may be due to the smaller number of samples.

Fig. 4 shows the changes of the three nitrogen fractions in brain slices incubated with and without glucose. The increase of ammonia in the absence of glucose accounts for a larger proportion of the newly formed NPN than that which is accounted for by the sum of ammonia and amide N in the presence of glucose.

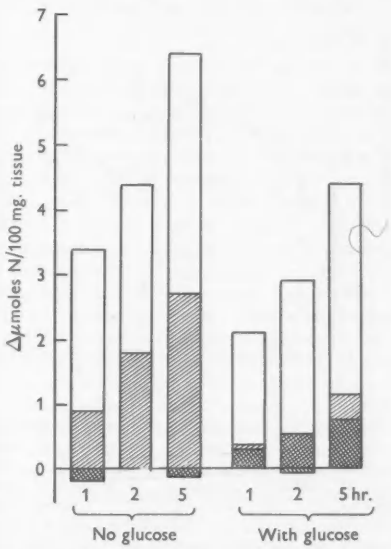


Fig. 4. Changes of non-protein N, ammonia and amide N during incubation of guinea-pig brain slices, in the presence and absence of glucose. Non-protein N, whole column, measured from 0; ammonia, shaded part of column; amide N, cross-hatched areas.

*Acid-soluble phosphorus.* The changes of acid-soluble phosphorus during the first 2 hr. of incubation are slight (Fig. 5). In both media an initial decrease is observed, which is, however, not significant. Later there is a rise which, after 5 hr., reaches a value significantly higher in the glucose-free than in the glucose-containing medium ($P<0.01$). Although, in the absence of glucose, the increase after 5 hr. is significant whether compared with the initial or the 30 min. value ($P<0.01$), in the presence of glucose only the difference between the 30 min. and the 5 hr. values is significant ($P=0.02$).

*Lipid and nucleic acid phosphorus.* After the incubation of brain slices in a glucose-free medium for 5 hr. a small, but probably significant, decrease occurs in the lipid-P fraction (see also Sperry, 1947) and a relatively larger and highly significant decrease in the ribonucleic acid (RNA) fraction (Table 3). With glucose present the changes are

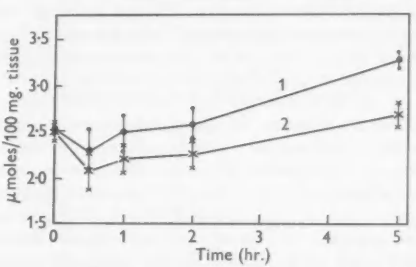


Fig. 5. Acid-soluble phosphorus in guinea-pig brain slices. Curve 1 (●—●), without glucose (initial and final values are means of 13 expts., intermediate values means of 5 expts., with S.E.); curve 2 (×—×), with glucose (initial and final values are means of 12 expts., intermediate values means of 5 expts., with S.E.).

Table 3. *Changes in lipid, nucleic acid and protein fractions of guinea-pig brain slices during an incubation of 5 hr. in the presence and absence of glucose* (0·2 %)

| | Glucose | No. of observations | μmoles/100 mg. tissue Initial* | Final* | Difference | *P* (probability factor) |
|---|---|---|---|---|---|---|
| Lipid P | Absent | 9 | 6·45±0·156 | 6·08±0·081 | −0·37 | 0·05 |
| | Present | 7 | 6·21±0·105 | 5·86±0·151 | −0·35 | 0·08 |
| RNA P | Absent | 9 | 1·13±0·020 | 0·858±0·0086 | −0·27 | <0·01 |
| | Present | 7 | 1·17±0·021 | 1·08±0·043 | −0·09 | 0·07 |
| DNA P | Absent | 9 | 0·223±0·0227 | 0·180±0·0106 | −0·04 | 0·12 |
| | Present | 7 | 0·214±0·0086 | 0·210±0·0118 | −0·004 | >0·2 |
| Protein N | Absent | 8 | 91·3±3·68 | 80·4±3·55 | −10·9 | 0·05 |
| | Present | 7 | 89·8±2·96 | 82·5±3·59 | −7·3 | 0·15 |

* Mean with S.E.

smaller and no longer significant. The deoxyribonucleic acid (DNA) fraction remains unchanged in both media. Admittedly the so-called RNA fraction includes other phosphorylated compounds in addition to RNA, especially when the analysis is done on brain tissue (Logan, Mannell & Rossiter, 1952). Since only the acid-soluble P was estimated in the acidified alkaline digest, the fraction also includes phosphoproteins.

The last row of Table 3 contains figures for the protein N, calculated according to Leslie & Davidson (1951). The method consists in estimating the nitrogen content of the alkaline digest before acidification. 'Protein N' is obtained by subtracting the amount of N corresponding to the sum of RNA-P and DNA-P, the assumption being made that 1 g. nucleic acid P corresponds to 1·69 g. nucleic acid N. Although there are obvious objections to this procedure, mainly on account of the low specificity of the RNA estimation, the corrections are relatively small and are, in fact, of the same order of magnitude as the standard error of the mean of the protein N values. After an incubation of 5 hr. a decrease of the protein N is found with and without glucose, but this is only significant without glucose. Though small in relation to the size of the protein N, the decrease is large enough to account amply for the increase of NPN.

*Experiments with brain suspensions*

When rat brain is dispersed in a hypotonic $NaHCO_3$ buffer, the initial ammonia content is about 1·0–1·5 $\mu$moles/100 mg. tissue; it increases only slightly during subsequent incubation. When isotonic salt solution is used, the initial ammonia concentration is lower and subsequent aerobic incubation causes a significant increase. Under anaerobic conditions the curve is similar to that found with the 'water suspension' (Fig. 6). The results on the mean ammonia formation of guinea-pig brain slices have been included in Fig. 6 for comparison.

DNP has an inhibitory effect on the ammonia formation of a brain suspension in isotonic salt solution, but this is less pronounced than in the slice. On the assumption that the ammonia formation depends on oxidative phosphorylation, the effect of adding high-energy phosphate groups in the form of ITP was investigated. ITP was chosen rather than ATP since it does not liberate ammonia in contact with tissues; it is an effective phosphate donor owing to the presence of nucleoside diphosphokinase in tissues (Krebs & Hems, 1953; Berg & Joklik, 1954). ITP, far from increasing the ammonia formation of the isotonic suspension, inhibited it to an extent similar to the effect of DNP. No summation of effects occurred when the two substances were added together (Table 4).

The differences between the results obtained with slices and with 'isotonic' suspensions may to some extent be due to the larger admixture of white matter in the latter, but if this factor entirely accounted for them one would expect a closer qualitative resemblance of the two time curves (Fig. 6, nos. 1 and 4). In addition to those already

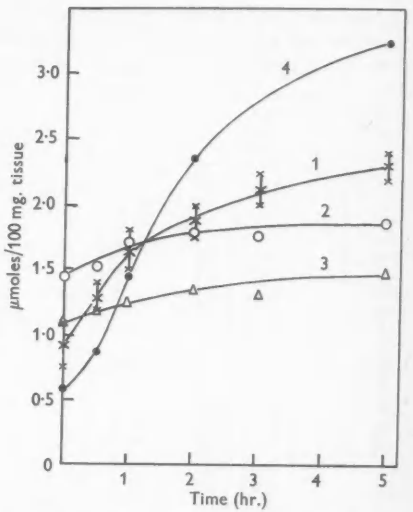


Fig. 6. Ammonia formation of brain preparations. Curve 1 (×—×), rat-brain dispersion in isotonic medium, aerobic (means of 5 expts. with S.E.); curve 2 (○—○), rat-brain dispersion in hypotonic medium (means of 2 expts.); curve 3 (△—△), rat-brain dispersion in isotonic medium, anaerobic (means of 3 expts.); curve 4 (●—●), guinea-pig brain slices (means of 13 expts.).

Table 4. *Effect of* 2:4-*dinitrophenol* ($10^{-3}$ M) *and inosine triphosphate* ($2·5 \times 10^{-3}$ M) *on the ammonia formation of an isotonic suspension of rat brain*

| Addition | $NH_3$ formed ($\mu$moles/100 mg. tissue) 0 | 1 hr. | 2 hr. | 4 hr. |
|---|---|---|---|---|
| None | 1·41 | 2·05 | 2·25 | 2·47 |
| ITP | 1·41 | 1·66 | 2·12 | 2·19 |
| DNP | 1·41 | 1·75 | 2·10 | 2·10 |
| ITP + DNP | 1·53 | 1·80 | 1·97 | 2·04 |

mentioned, other attempts were made to influence the ammonia formation of brain dispersions, but the following additions, singly or combined, were without effect: $MgCl_2$ (0·005 M), nicotinamide (0·04 M), diphosphopyridine nucleotide (6 $\mu$g./ml.),

NaF (0·02 M), cytochrome *c* ($0·5 \times 10^{-4}$ M). The basal medium in these experiments was the potassium-rich salt solution.

The formation of NPN during the incubation of brain dispersions was studied in isotonic medium under aerobic and anaerobic conditions (5 and 2 expts., respectively) and in hypotonic medium under aerobic conditions (2 expts.). The experiments lasted for 5 hr. and intermediate values were determined after 0·5, 1, 2 and 3 hr. No significant changes were observed, all values lying in the range 10·8–13·0 $\mu$moles/100 mg. tissue.

*Experiments with washed or dialysed preparations*

Many of the components that make up the NPN fraction of brain, including 15 L-amino acids (alanine, arginine, aspartate, cysteine, glutamate, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine and valine, all at 0·01 M; cystine, saturated soln.; glutathione (–SH), 0·01 M), were tested for their ability to form ammonia in experiments with dialysed or washed brain suspensions. A significant ammonia formation was obtained only from the following: ATP, ADP, adenylic acid, adenosine, L-glutamine and L-glutamic acid. This agrees with what is known of the deaminating systems occurring in brain. Various combinations of substrates, especially amino acids, were also tried, but except for the combination aspartic acid–glutamic acid no significant effects were found. Aspartic acid slightly increased the ammonia formation from glutamic acid, an effect which can be explained by the transamination reaction. Since these experiments did not contribute anything to our understanding of the ammonia formation of brain slices they are not reported in detail.

It was observed during these experiments that the addition of ITP greatly increased the ammonia formation from glutamine. A combination of inosinic acid and creatine phosphate had the same effect, whereas either of these substances alone had little or no activity. This was at first interpreted as indicating a reamination of inosine phosphate or polyphosphate by glutamine and subsequent deamination by adenylic deaminase (Weil-Malherbe, 1953*a*). It was later found, however, that the effect disappeared when a phosphate buffer was substituted for the bicarbonate buffer previously used. Presumably the effect was due to the liberation of phosphate from ITP and to the well-known stimulation of glutaminase by inorganic phosphate (Errera & Greenstein, 1949).

It was further found that washed rat-brain mitochondria possessed high glutaminase and adenylic deaminase activities.

## DISCUSSION

These results show that the ammonia formation of brain tissue *in vitro* has two components, one that is presumably mainly autolytic, while the other depends on the structural and functional integrity of the cell or its elements. The structural integrity of subcellular elements is probably better preserved in the isotonic suspension than in the 'water suspension', though of course not so well as in the slice. The ammonia formation of the isotonic suspension is therefore intermediate between those of the other two preparations. The functional integrity of the cell is impaired by anoxia, respiratory inhibitors or 'uncoupling' agents; under these conditions the ammonia formation of slices and isotonic suspensions is lowered to the level observed in water suspensions. Although the ammonia content of brain *in vivo* is only about 0·02–0·03 $\mu$mole/100 mg. (Schwarz & Dibold, 1932; Richter & Dawson, 1948) the initial value found in water suspensions was about 1·5 $\mu$moles/100 mg. In slices the initial value was about 0·5 $\mu$mole/100 mg., although the time interval since the death of the animal was longer than in the experiments with suspensions. This suggests that in the slice, and to some extent in the isotonic suspension, the autolytic processes are inhibited.

The following five deaminating enzyme systems are known to occur in brain: glutaminase, glutamic dehydrogenase, amine oxidase, adenylic deaminase and adenosine deaminase. These five enzyme systems and their possible implication in the ammonia formation of brain slices in a substrateless medium will now be considered.

*Glutaminase.* It has previously been shown (Weil-Malherbe, 1936) and confirmed in the present investigation that the amide concentration remains fairly constant in brain slices, even under anaerobic conditions. In any case the amount of preformed amide N could account for only a small proportion of the ammonia evolved. The fact that the ammonia formation of brain slices is unaffected by methionine sulphoxide as well as by D-glutamic acid, one of which inhibits glutamine synthesis and the other glutamine hydrolysis, seems to exclude the possibility of a cycle of glutamine synthesis and deamination.

*Glutamic dehydrogenase.* It is known (Krebs, 1935; Weil-Malherbe, 1936) that during the oxidation of added L-glutamic acid by brain slices no free ammonia arises, owing to the simultaneous synthesis of glutamine according to the reaction:

$$2 \text{ glutamic acid} + \tfrac{1}{2}O_2 \rightarrow \text{glutamine} + \alpha\text{-oxoglutaric acid.}$$

The addition of oxoglutaric acid results in an inhibition of ammonia formation and, if glucose be

present, even in the disappearance of added ammonium ion (Weil-Malherbe, 1936). This is due to the fact that the equilibrium of the glutamic dehydrogenase reaction is strongly in favour of the reductive amination of oxoglutaric acid (Euler, Adler, Günther & Das, 1938; Strecker, 1953). It is also unlikely that a cycle of transamination, with oxoglutaric acid as amino acceptor, is involved in the ammonia formation of brain slices, since in that case the addition of oxoglutaric acid might be expected to increase rather than decrease the evolution of ammonia. Finally, the fact that the ammonia formation of isotonic suspensions was unaffected by the addition of nicotinamide or of nicotinamide + diphosphopyridine nucleotide also argues against the involvement of glutamic dehydrogenase, in view of the rapid destruction of preformed diphosphopyridine nucleotide in such a preparation (McIlwain & Rodnight, 1949).

*Amine oxidase.* A survey of the nitrogenous components of deproteinized brain extracts (Weil-Malherbe, 1953*b*) shows that substrates of this enzyme occur only in minute amounts. Moreover, its inhibitor, ephedrine, is without effect on the ammonia formation of brain slices.

*Adenylic and adenosine deaminases.* It has been suggested by Embden and later by Parnas (see, for example, Parnas & Lewinski, 1935; Parnas & Lutwak-Mann, 1935) that the ammonia formation of working muscle is due to the activity of adenylic deaminase. This theory has been widely accepted, in spite of the fact that the agreement between the disappearance of adenosine phosphates and the formation of ammonia is not quantitative, since the sum of adenosine nucleotide amino-N and free $NH_3$ increases (Parnas & Lutwak-Mann, 1935). To explain this, the assumption had to be made that adenylic acid was taking part in a deamination–reamination cycle. Kalckar & Rittenberg (1947) found a rapid incorporation of $^{15}N$ into the amino group of muscle adenosine nucleotides when ammonium citrate labelled with $^{15}N$ was fed to rats and they interpreted this as confirmation of the existence of such a cycle. The possibility, however, has not been considered that the effect might be due to an exchange reaction between the adenylic amino group and free ammonium ions, catalysed by adenylic deaminase, in analogy to the exchange reactions catalysed by glutamine synthetase (Grossowicz, Wainfan, Borek & Waelsch, 1950; Stumpf, Loomis & Michelson, 1951; Elliott, 1953; Levintow & Meister, 1954). Wajzer & Nekhorocheff (1952) recently found an increase of the adenine fraction and a somewhat smaller disappearance of the hypoxanthine fraction during aerobic recovery of isolated frog muscle stimulated *in vitro*. Nekhorocheff & Wajzer (1952) and Nekhorocheff (1952) further claim that the reamination of inosinic acid occurs at the expense of the $-NH.PO_3H_2$ group of creatine phosphate, which is itself converted into urea. We have been unable to demonstrate any increased formation of ammonia after the addition of ITP or inosinic acid, with or without creatine phosphate, to brain slices or brain dispersions, washed, dialysed or untreated. In the absence of more definite evidence for the occurrence of the reamination reaction in brain it would be purely speculative to assume that the ammonia formation of brain slices involves a deamination–reamination cycle of adenosine nucleotides.

The concentration of preformed adenosine nucleotides in brain is of course far too small to account for the total of even the 'autolytic' fraction of the ammonia formation. According to the highest estimates their concentration is about 0·33 $\mu$mole/100 mg. in the rat (Lindberg & Ernster, 1950) and the guinea pig (Kratzing & Narayanaswami, 1953), while with more specific enzymic methods a value of about 0·17 $\mu$mole/100 mg. was obtained for rat brain (Albaum & Chinn, 1953). According to Tsukada & Takagaki (1954) the adenylic compounds did not decrease in guinea-pig brain slices during an incubation of 30 min.

We are thus led to the conclusion that there is no evidence which would allow one to ascribe the ammonia formation in brain slices to any of the five known deaminating systems occurring in brain.

In agreement with Astrup, Carlström & Stage (1951) we found no increase in the NPN of brain dispersions during an incubation of 5 hr. On the other hand, a significant increase was observed upon incubation of brain slices. This reaction therefore depends on the integrity of cellular structure in a way similar to the non-autolytic ammonia formation. Ansell & Richter (1954) have recently reported the existence of a very unstable neutral proteinase in brain which could not be extracted and which was only active for a short time after death. It is suggested, at present merely as a working hypothesis, that the ammonia formation of brain slices is connected with the activity of this enzyme.

The question as to whether the ammonia formation of brain slices is related to the ammonia formation induced by the stimulation of nervous tissues cannot be answered as yet. Geiger, Dobkin & Magnes (1953) have found that stimulation of cat brain cortex led to a marked increase of NPN. This would be in favour of a close connexion between the two processes. On the other hand, it may be pointed out that a similar and probably related process of ammonia formation occurs in other than nervous tissues, such as kidney, spleen, bone marrow, embryonic and neoplastic tissues (Warburg *et al.* 1924; Dickens & Greville, 1933; Lutwak-Mann, 1951). Most of these have a high glycolytic capacity (cf. Warburg *et al.* 1924).

The virtually complete suppression of the ammonia formation of brain slices in a glucose-containing medium still requires some comment. It is partly due to a lower degree of proteolysis, as shown by the NPN curves. The formation of acid-soluble phosphate and the fall of RNA-phosphate are similarly decreased in the presence of glucose. Glucose has therefore an inhibitory effect on catabolic reactions. The second factor is the operation of an ammonia-binding mechanism. Brain slices in glucose–saline form a considerable amount of amide N, no doubt at the expense of ammonium ions formed in a primary reaction. If the amide was formed by combination of ammonium ions with oxoglutaric acid, two moles of ammonium ions are fixed for each mole of amide N. Since an additional fraction of free ammonia may have disappeared in other reactions, e.g. in amination of oxoglutaric acid followed by transaminations to pyruvic or oxaloacetic acids, it is likely that, in relation to the amount of proteolysis, the primary ammonia formation is the same in the presence as in the absence of glucose.

## SUMMARY

1. Slices of rat or guinea-pig brain in a glucose-free medium form ammonia at a fairly steady rate for at least 5 hr. During this period a final concentration of 3–4 $\mu$moles/100 mg. tissue is reached.

2. The concentration of amide nitrogen does not change significantly during the aerobic or anaerobic incubation of brain slices.

3. The ammonia formation of brain slices is suppressed in glucose–saline. Instead, a synthesis of amide nitrogen takes place.

4. The ammonia formation of brain slices in glucose-free medium is inhibited under anaerobic conditions and by those agents that interfere with electron transport or oxidative phosphorylation.

5. The non-protein nitrogen and, to a lesser extent, the acid-soluble phosphorus of brain slices increase significantly during an incubation of 5 hr., particularly in a glucose-free medium.

6. A small, but probably significant, decrease occurs in the lipid phosphorus fraction and a relatively larger decrease in the ribonucleic acid phosphorus fraction of brain slices maintained in glucose-free medium. In glucose–saline the changes are smaller and not significant. The deoxyribonucleic acid phosphorus remains unchanged in the absence as well as in the presence of glucose.

7. Compared with brain slices, the initial ammonia concentration is higher and the final concentration lower when brain suspensions are incubated. In a hypotonic medium very little ammonia is formed upon incubation. In isotonic medium some ammonia is formed aerobically, but not anaerobically. The addition of high-energy phosphate groups or of diphosphopyridine nucleotide is without effect on ammonia formation. No increase of non-protein nitrogen was found in brain suspensions.

8. Potential parent compounds were studied as to their effect on the ammonia formation of washed or dialysed brain suspensions and of brain mitochondria. These studies contributed nothing to the understanding of the mechanism of ammonia formation in slices.

9. It is concluded from the results that none of the five known deaminating brain enzymes is a major factor in the ammonia formation of brain slices and it is suggested that the ammonia arises in a reaction intimately linked with proteolysis.

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# Ammonia Formation in Brain

## 2. BRAIN ADENYLIC DEAMINASE

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During our studies on the ammonia formation of brain slices (Weil-Malherbe & Green, 1955) the possibility was envisaged that adenylic deaminase might be the terminal enzyme, acting perhaps as a component of a reamination–deamination cycle. The study of this enzyme was originally undertaken in the hope that it might yield evidence bearing on this hypothesis.

Muntz (1953) recently described an unusual type of adenylic deaminase in aqueous extracts from acetone-dried dog brain. The enzyme was strongly activated by adenosine triphosphate, an effect that was greatest when the concentrations of adenosine triphosphate and adenylic acid were equal. The ammonia formation from adenylic acid alone was interpreted as being secondary to the conversion of adenylic acid into adenosine.

In the present paper observations on the purification of this enzyme and on the properties of the purified preparation are reported.

## EXPERIMENTAL

### *Methods*

*Abbreviations.* Adenosine triphosphate, adenosine diphosphate and 5′-adenylic acid are denoted by ATP, ADP and AMP. ITP, IDP and IMP stand for the corresponding inosine phosphates. Ethylenediaminetetraacetate is denoted EDTA and trichloroacetic acid TCA.

*Analytical.* Enzyme activity was measured either by the estimation of ammonia or spectrophotometrically. Filtrates deproteinized with TCA at a final concentration of 5% (w/v) were used for the ammonia estimations, which were carried out by the microdiffusion method (Conway, 1950) and nesslerization; in some experiments the filtrates were analysed by direct nesslerization. The spectrophotometric method is based on the decrease of extinction at 265 m$\mu$. and the increase at 240 m$\mu$. when adenine nucleotides are deaminated to hypoxanthine nucleotides. In a solution with a nucleotide content corresponding to 1 $\mu$g. adenine/ml. $\Delta E_{265\,m\mu.} = -0{\cdot}059$ and $\Delta E_{240\,m\mu.} = +0{\cdot}032$ (Kalckar, 1947). Since the enzyme preparations were turbid, the solutions were deproteinized with $HClO_4$ at a final concentration of 3% (w/v). The filtrates were diluted to contain $0{\cdot}3$–$1 \times 10^{-4}$M total nucleotides. A Unicam SP. 500 quartz spectrophotometer was used for the measurements.

Phosphate was estimated by a modification of the method of Martin & Doty (Weil-Malherbe & Green, 1951; Weil-Malherbe, 1953) and protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

*Standard conditions.* For activity measurements the enzyme was incubated in a solution containing 0·0033M AMP, 0·0033M ATP, 0·02M sodium succinate buffer (pH 6·2) and 0·01M sodium EDTA (pH 6·8), in a total volume of 1·5 ml. or multiples thereof. The final pH of the

mixture was about 6·7. The buffering capacity of succinate at this pH is slight, but enzyme activity was higher in this mixture than in other buffers (see below). Incubation was carried out in stoppered test tubes at 30° for 30 or 60 min.

Other experimental procedures are described in the following section.

*Materials.* ATP was prepared by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948) and purified by ion-exchange chromatography (Cohn & Carter, 1950). ADP was prepared by incubating ATP with glucose and purified yeast hexokinase (fraction 3*a* of Berger, Slein, Colowick & Cori, 1946). For ITP and creatine phosphate see Weil-Malherbe & Green (1955). IDP was prepared from ADP by treatment with $HNO_2$. (Found: total organic P, 8·1; acid-labile P, 4·31; inorganic P, 0·15%; N, 7·81; pentose 20·0%; molecular extinction coefficient at 250 mμ. 12400. Calc. for $C_{10}H_{11}O_{11}N_4P_2Ba_{1·5},6H_2O$: total P, 8·39; acid-labile P, 4·20; N, 7·59; pentose, 20·3%.)

AMP was obtained from Zellstoff-fabrik Waldhof, Wiesbaden, Germany, IMP from Boots Pure Drug Company, Nottingham.

## *Results*

*Purification.* The purification of brain adenylic deaminase turned out to be more difficult than anticipated, mainly on account of the instability of the enzyme. It loses activity after some days, even in the cold, and especially after partial purification. There were, however, exceptions and some fractions remained active for 2–3 weeks.

content. After centrifuging, almost all of the activity remained in the supernatant. It was found, however, that the activity was still associated with particulate matter which could be spun down completely at 15 000 *g*. The high-speed centrifugate contained only traces of protein (<0·1 mg./ml.). A freshly prepared high-speed residue, suspended in 0·1 M succinate buffer (pH 6·2) (one-third of the volume before high-speed centrifuging), was used for most experiments. This fraction, which is henceforth referred to as the 'particulate fraction', could be freeze-dried and, though some activity was lost, the resulting material was comparatively stable.

In the following the details of an actual preparation are given. 40 g. acetone powder were stirred in 400 ml. ice-cold glass-distilled water for 15 min. The mixture was centrifuged in an angle centrifuge (MSE Angle Head 6886, Measuring and Scientific Equipment Ltd., London) for 30 min. at 4000 rev./min. and 3°. The supernatant was again centrifuged for a further period of 2 hr. The slightly turbid solution (220 ml.) was mixed with 94 ml. saturated $(NH_4)_2SO_4$ solution. After centrifuging, the precipitate was dissolved in a small volume of water. The resulting solution was 8% saturated with respect to $(NH_4)_2SO_4$. Saturation was increased to 40% by the addition of solid $(NH_4)_2SO_4$ (19 g./100 ml.). The precipitate was centrifuged off, dissolved in glass-distilled water and dialysed at 3° against 10 l. running glass-distilled water over a period of 48 hr. The dialysed solution (130 ml.; pH 6·40) was adjusted to pH 7·0. A portion of 20 ml. was mixed with 8 ml. alumina gel C*γ* (320 mg. dry wt./10 ml.) and shaken for 5 min. The gel was removed by a short centrifuging at 1500 *g*. The supernatant was now centrifuged for 15 min. in the MSE Superspeed unit at 15 000 *g* and the residue suspended in 7 ml. succinate buffer.

Table 1. *Preparation of purified adenylic deaminase from 40 g. of acetone-dried powder of ox brain*

The activity tests were carried out under standard conditions for 30 min.

| Fraction | Protein content of fraction (mg.) | Activity (μg. $NH_3$/mg. protein) |
|---|---|---|
| Aqueous extract of powder | 1660 | 7·8 |
| After $(NH_4)_2SO_4$ fractionation | 415 | 31·4 |
| After treatment with alumina gel C*γ* | 79 | 133 |

Table 2. *Adenylic deaminase activity of extracts of brain acetone powders prepared from grey and white matter separately*

| Nucleotides added (0·0033 M) | μg. $NH_3$/5 mg. protein/hr. | |
|---|---|---|
| | Grey matter | White matter |
| AMP | 13·9 | 8·4 |
| ATP | 2·8 | 3·5 |
| AMP + ATP | 67·6 | 72 |

Since the activity tended to deteriorate, a simple and rapid procedure had to be adopted. It consisted essentially of two steps: a fractionation with ammonium sulphate and a treatment with aluminium hydroxide gel C*γ* (Willstätter & Kraut, 1923, 1924). Acetone-dried ox brain was the starting material. The aqueous extract was brought to 30% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved and reprecipitated by $(NH_4)_2SO_4$ at 40% saturation. The fraction was thoroughly dialysed against running distilled water and was then shaken with sufficient alumina C*γ* at pH 7·0 to adsorb about 80% of the protein

The purification factor attained in this preparation was 16·9 (Table 1). It varied in other experiments between 10·2 and 21·3. 81% of the original activity was recovered.

Crude aqueous extracts had the same activity whether they were prepared from the acetone-dried grey or white matter of ox brain (Table 2). The whole brain was therefore used in the preparation of the acetone powder.

*Solvent fractionation.* The aqueous extract of brain acetone powder could also be fractionated by acetone or ethanol precipitation, but only with considerable losses of activity. The highest activity was found in the fractions precipitated between 20 and 30% ethanol (v/v) and between 30 and 40% acetone (v/v), but in both cases only about 25% of the original activity were recovered.

*Solubilization.* Attempts were made to solubilize the enzyme by treatment with *n*-butanol, according to Morton (1950). The solvent was stirred with 3 vol. enzyme solution either at 30° or at 0° and the activity of the aqueous layer

tested after dialysis. The enzyme solutions thus treated were water or buffer extracts of acetone powder or fractions precipitated by $(NH_4)_2SO_4$. In every case the enzyme was found to have been inactivated. In another experiment a freeze-dried particulate fraction was suspended in *n*-butanol at $-20°$. The mixture was centrifuged at $-10°$ and the residue dried at $-10°$ *in vacuo* (0·1 mm. Hg). The dry powder was extracted with 0·1 M succinate buffer and centrifuged at 15 000 *g*. Though the precipitate was still active, no activity had passed into solution.

In a further series of experiments the particulate fraction, in samples of 0·5–1 mg. protein, was suspended in 1·5 ml. of a solution containing buffer and a bile salt and stirred for 15 min. at room temperature. After centrifuging at 15 000 *g* for 15 min. in the cold, the supernatant and insoluble fractions were tested separately, the latter after resuspension in the original volume of buffer solution. A suspension, treated with bile salt, but not centrifuged, and another, not treated with bile salt, served as controls.

Sodium deoxycholate was used at concentrations of 3, 1, and 0·33 % (w/v). It was highly inhibitory at all levels. No activity was found in any of the supernatant fractions and only with the lowest concentration was any activity found in the insoluble fraction. Results were similar with sodium tauroglycocholate. With sodium cholate, on the other hand, some solubilization apparently occurred, as shown by the appearance of activity in the supernatant fractions (Table 3). The inhibitory effect of the bile salt is obvious from its effect on the whole suspension. In the absence of the bile salt the sum of the activities of the soluble and insoluble fractions accounted for that of the whole suspension, but in its presence the activities of the centrifuged fractions exceeded the activity of the whole suspension, a discrepancy that increased with increasing bile salt concentration. This is due to the fact that the insoluble fraction was resuspended in buffer solution free from inhibitor.

*Presence of other enzymes in the particulate fraction.* As shown in Table 4, the particulate fraction possesses some 5-nucleotidase and ATPase activity in the presence of $Mg^{2+}$, though very little in its absence. Adenosine deaminase activity is also very slight. The ammonia formation found when AMP is the only substrate, therefore, cannot be accounted for by adenosine deaminase activity, as Muntz had assumed; moreover, the ammonia formation from AMP in the Mg-free medium was 9 times higher than the formation of inorganic phosphate. The particulate fraction also had weak myokinase activity, but this was apparent only in a medium containing glutathione and histidine (Table 5). Under standard conditions myokinase activity was practically absent.

*The activating effect of ATP.* The effect of an equimolar amount of ATP on the deamination of 5 μmoles AMP is illustrated in Fig. 1. In this experiment about 20 min. were required for 50 % deamination in the presence of ATP, as compared with 3·3 hr. in its absence. In the absence of ATP the deaminating activity varied, but never disappeared completely (see also Tables 4 and 7); as it was unaffected by repeated washing or by dialysis of the particulate fraction, it is unlikely that it can be attributed to traces of ATP in the preparation. On the other hand, fractionation of the crude brain extract yielded preparations in which the activation effect of ATP was slight or lacking, without nucleoside being formed at a sufficient rate to implicate adenosine deaminase (Table 6). It may be assumed therefore either that there are two different adenylic deaminases, one responsive to the activating effect of ATP and the other not, or that the effect of ATP requires an additional factor that was absent or inactive in some fractions. By contrast, the treatment with alumina gel greatly enhanced the activating effect of ATP while leaving the activity without ATP unchanged.

Table 3. *Solubilization of adenylic deaminase by sodium cholate*

| Sodium cholate concn. (g./100 ml.) | Protein (mg.) | Activity of fractions (μg. $NH_3$/50 min.) | | |
|---|---|---|---|---|
| | | Not centrifuged | Supernatant | Insoluble (resuspended) |
| 0 | 0·16 | 53·0 | 1·8 | 49·5 |
| 0·25 | 0·16 | 35·8 | 16·8 | 26·8 |
| 0·5 | 0·16 | 26·8 | 16·8 | 18·3 |
| 1·0 | 0·21 | 10·8 | 7·3 | 15·0 |

Table 4. *Enzyme activities of particulate fraction*

Medium *A*: 0·06 M sodium glycylglycine buffer (pH 7·1), 0·005 M-$MgCl_2$, 0·005 M-KCl. Medium *B*: 0·05 M sodium succinate buffer (pH 6·2), 0·01 M EDTA, 0·005 M-KCl. Concentration of substrates: 5 μmoles/1·5 ml. (0·0033 M).

| Substrates added | Formation of inorganic phosphate | | Formation of $NH_3$ |
|---|---|---|---|
| | Medium *A* | Medium *B* | Medium *B* |
| | (μmoles/mg. protein/hr.) | | |
| Adenosine | 0 | 0 | 0·14 |
| AMP | 1·03 | 0·13 | 1·20 |
| ATP | 2·33 | 0·40 | 0·25 |
| AMP + ATP | 1·70 | 0·50 | 6·50 |

Table 5. *Myokinase activity of particulate fraction*

Medium *A*: 0·01 M sodium succinate buffer, 0·005 M glucose, 0·067 % (w/v) glutathione, 0·0067 M histidine, 0·0067 M-$MgCl_2$, 10 μg. yeast hexokinase/ml. Medium *B*: 0·033 M sodium succinate buffer, 0·005 M glucose, 0·0067 M-$MgCl_2$, 10 μg. yeast hexokinase/ml. Medium *C*: as medium *B*, but with 0·01 M EDTA instead of $MgCl_2$. All tests contained 5 μmoles ADP in 3 ml. (0·00167 M).

| | Δ Acid-labile phosphate | Δ Glucose |
|---|---|---|
| | (μmoles/mg. protein/30 min.) | |
| Medium *A* | −0·75 | −0·8 |
| Medium *A*, without yeast hexokinase | 0 | −0·2 |
| Medium *B* | −0·21 | 0 |
| Medium *C* | −0·16 | 0 |

Table 6. *Effect of enzyme fractionation on the formation of ammonia and inorganic phosphate*

Standard conditions. Substrates at 0·0033 M concentration.

| In presence of ... ... | $NH_3$ formation | | | Phosphate formation |
|---|---|---|---|---|
| | AMP + ATP | AMP | Adenosine | AMP |
| Fractions | ($\mu$moles/mg. protein) | | | |
| $(NH_4)_2SO_4$ 20–30% sat. | 2·94 | 0·52 | 0·55 | 0·12 |
| 30–35% sat. | 0·54 | 0·34 | 0·62 | 0·12 |
| 40–50% sat. | 0·32 | 0·32 | 1·52 | 0·12 |
| Ethanol 20–30% (v/v) | 1·70 | 0·53 | 0·55 | 0·15 |
| 30–40% (v/v) | 0·41 | 0·50 | 0·41 | 0·14 |
| $(NH_4)_2SO_4$ 0–40% sat. | 1·26 | 0·63 | 0·36 | — |
| Particulate fraction | 6·65 | 0·64 | 0 | — |

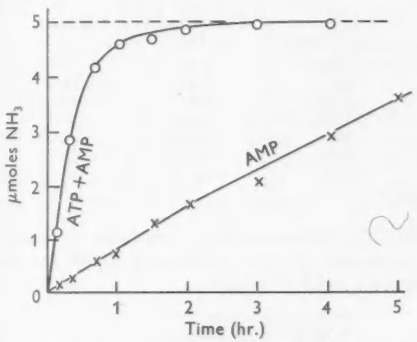


Fig. 1. Time curve of ammonia formation by particulate adenylic deaminase fraction. Nucleotides: 5 $\mu$moles/1·5 ml.

Table 7. *Activation of adenylic deaminase by various nucleotides*

0·4 mg. protein (particulate fraction)/1·5 ml. Concentration of nucleotides: 0·0033 M.

| Addition | $NH_3$ ($\mu$g.) |
|---|---|
| AMP | 3·0 |
| AMP + ATP | 42·6 |
| AMP + ADP | 6·6 |
| AMP + ITP | 5·4 |
| AMP + IDP | 4·5 |

The effect of ATP is specific. Replacement of ATP by ADP, ITP or IDP only led to insignificant activation (Table 7). This fact is demonstrated much more clearly with the purified enzyme than with the preparations used by Muntz. In a crude dialysed aqueous extract of brain acetone powder ADP had the same activating effect as ATP; it was only slightly decreased in a Mg-free medium where myokinase was strongly inhibited, as shown by the inhibition of the ammonia formation from ADP (Table 8). Creatine phosphate, in the presence of $Mg^{2+}$, inhibits the

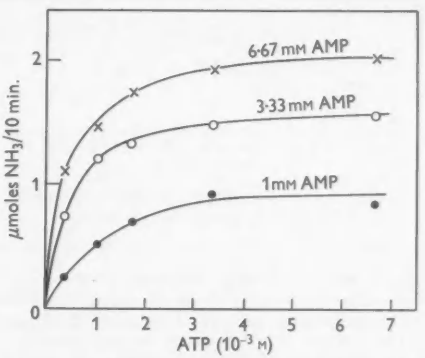


Fig. 2. Effect of ATP at varying concentrations of AMP on adenylic deaminase activity. Activity measured by ammonia formation in first 10 min.

Table 8. *Effect of* $Mg^{2+}$ *and EDTA on ammonia formation by dialysed aqueous extract of brain acetone powder* (1 *in* 10)

0·3 ml. extract/1·5 ml., 30 min.

| Additions (0·0033 M) | With 0·005 M-$MgCl_2$ | With 0·01 M EDTA | Effect of absence of $Mg^{2+}$ |
|---|---|---|---|
| | ($\mu$moles $NH_3$) | | |
| ATP | 1·0 | 0·13 | Inhibition of ATPase |
| ADP | 1·22 | 0·18 | Inhibition of myokinase |
| AMP | 1·5 | 1·28 | — |
| ATP + AMP | 4·6 | 4·6 | — |
| ADP + AMP | 4·6 | 4·12 | — |
| AMP + creatine phosphate | 0·52 | 1·2 | Inhibition of creatine phosphokinase and/or myokinase |
| ATP + AMP + creatine phosphate | 0·9 | 4·6 | |

deamination of AMP and its activation by ATP, no doubt owing to competition of creatine phosphokinase. The effect is suppressed if $Mg^{2+}$ is absent.

According to Muntz the highest activation is obtained when ATP and AMP are present in equal concentration. At a low concentration of AMP maximum activation should therefore be observed at lower ATP concentrations than at a high concentration of AMP. However, when care is taken to measure the reaction rate during the near-linear phase, activity/concentration curves of ATP are obtained that are of a similar shape at different levels of AMP concentration, reaching a maximum between 3 and 5 mM ATP (Fig. 2).

pH *Optimum.* The pH/activity curve of the enzyme has a broad plateau between pH 6·2 and 6·8, when measured in pyrophosphate buffer, both with and without ATP (Fig. 3). This is a little more towards the acid side than the optimum found by Muntz.

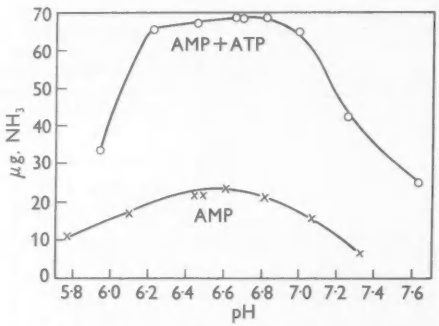


Fig. 3. Effect of pH on activity of adenylic deaminase in 0·02 M sodium pyrophosphate buffers.

*Effect of cations and anions.* Replacement of $Na^+$ by $K^+$ did not affect the activity of the enzyme. On the other hand, the multivalent cations that were tested inhibited it, with the exception of $Ca^{2+}$ (Table 9). The addition of EDTA was, of course, omitted in these experiments.

Among anions, phosphate was found to have a marked inhibitory effect, in contrast to arsenate which inhibited only slightly. Pyrophosphate, too, caused some inhibition (Table 10).

*Other inhibitors.* The enzyme was inhibited by fluoride, *p*-chloromercuribenzoate and octan-2-ol (Table 11), but not by the following substances: arsenite, azide, cyanide, hydroxylamine, iodoacetate, 2:4-dinitrophenol, sulphanilamide (all at $10^{-3}$ M), DL-methionine sulphoxide (mixed diastereoisomers), benzaldehyde, ferrocyanide and urethane (at $10^{-2}$ M). Neither is the reaction inhibited by the end products, IMP or ammonia, at 0·01 M concentration, in the presence or absence of ATP.

*End products.* It is evident from Fig. 1 that the ammonia formation quantitatively accounts for the deamination of one of the two compounds present, AMP or ATP. Very little inorganic phosphate is formed under standard conditions (Table 4). Analysis of the final reaction mixture by paper chromatography and paper electrophoresis has shown that AMP is converted into IMP, while ATP is recovered unchanged.

It was found that paper chromatography with a solvent containing ethanol (75 vol.) and M ammonium acetate (pH 7·5) (30 vol.) (Paladini & Leloir, 1952) brought about a good separation of ATP ($R_F$ 0·37) and AMP ($R_F$ 0·61), but not of AMP and IMP. A solvent containing 100 ml. *iso*-butyric acid, 60 ml. N-$NH_3$ and 1·6 ml. 0·1 M EDTA (Krebs & Hems, 1953), on the other hand, satisfactorily separated IMP ($R_F$ 0·38) and AMP ($R_F$ 0·64), but not ATP and IMP.

Table 9. *Inhibition of adenylic deaminase activity of particulate fraction by cations*

Standard conditions.

| Additions | Concentration (M) | Inhibition (%) |
|---|---|---|
| $CaCl_2$ | 0·01 | 0 |
| $MgCl_2$ | 0·01 | 21 |
| $MnSO_4$ | 0·01 | 24 |
| $CoSO_4$ | 0·002 | 50 |
| $CuSO_4$ | 0·002 | 67 |
| $ZnSO_4$ | 0·002 | 57 |
| $FeCl_3$ | 0·002 | 48 |

Table 10. *Relative activity of adenylic deaminase of particulate fraction in various buffer solutions* (0·02 M, pH 6·69 ± 0·05)

| | |
|---|---|
| Sodium succinate | 100 |
| Sodium borate–succinate | 91 |
| Sodium acetate–veronal | 82 |
| Sodium arsenate | 82 |
| Sodium pyrophosphate | 70 |
| Sodium phosphate | 52 |

Table 11. *Inhibition of the adenylic deaminase activity of particulate fraction*

| | Concentration (M) | Inhibition (%) |
|---|---|---|
| NaF | 0·01 | 71 |
| *p*-Chloromercuribenzoate | 0·001 | 65 |
| Octan-2-ol | Saturated soln. | 42 |

A method was therefore devised in which a first run was carried out with the ethanol–ammonium acetate solvent in the descending direction for 18 hr. The spots were located by the photoprint method (Markham & Smith, 1949, 1951) and the paper strip was cut across between the ATP spot and the confluent spots corresponding to AMP and IMP. The distal part of the paper carrying the AMP and IMP spots was now subjected to a second run with ammonium *iso*butyrate solvent in the ascending direction for 20 hr. The spots, which by now were well separated, were again located by the photoprint method. The absorbing areas, including that cut off after the first run, were cut out and eluted twice with 5 ml. water. The extinction of the eluates was measured at 250 and 260 mμ. and the concentration of the nucleotides was calculated, a molecular extinction coefficient of 15 800 at 260 mμ. being assumed for AMP and ATP (Kalckar, 1947) and one of 12 300 at 250 mμ. for

IMP (Schulman & Buchanan, 1952). The ratio $E_{250}/E_{260}$ which is 1·60 for IMP and 0·83 for AMP and ATP was determined as an indicator of identity.

Samples of 1 ml. were withdrawn from the standard reaction mixture at the beginning and the end of the incubation and delivered into 1 ml. 10% (w/v) TCA. The filtrate was extracted 4 times with ether, the ether removed *in vacuo* and the volume made up to 2 ml. Samples of 0·3 ml., in portions of about 5 µl., were applied to the paper (Whatman no. 1, 48 × 8 cm., pretreated according to Eggleston & Hems, 1952). Neglecting the fraction of the solution lost during the filtration the amount of each of the two nucleotides applied to the paper strip was 0·5 µmole.

On the paper strip carrying the initial sample, two spots were located which occupied positions corresponding to ATP and AMP; on the strip carrying the final sample again only two spots were found in the positions of ATP and IMP, while there was no absorption corresponding to AMP. The results of the spectrophotometric analysis of the eluates are shown in Table 12. They confirm that ATP was unchanged, whereas AMP was quantitatively converted into IMP.

Identical results were obtained by the method of paper electrophoresis. The EEL paper-electrophoresis apparatus (Evans Electroselenium Ltd., Harlow, Essex), which was slightly modified so as to take a single paper strip of 35 × 15 cm., was used, with 0·02 M sodium citrate buffer (pH 4·7). An initial and a final sample were applied to the paper (Whatman no. 3 MM) in two separate spots at the cathode end of the strip in the same way as in the paper-chromatography experiments; a current of 9–10 mA (about 10 v/cm.) was applied for 6 hr. The spots were located by the photoprint method, cut out, eluted and estimated spectrophotometrically, as before. A result is shown in Table 13.

## DISCUSSION

The results obtained with a purified enzyme preparation on the whole confirm the conclusions reached by Muntz (1953) on the basis of experiments with crude extracts. They show that brain contains an adenylic deaminase which is strongly activated by ATP. However, ATP does not appear to be an indispensable cofactor, since the enzyme possessed some activity even in the complete absence of ATP. No indication was found for a stoicheiometric requirement for ATP; the mechanism by which ATP accelerates the reaction is obscure, as it is recovered unchanged. It is unlikely that the effect can be attributed to the chelating properties of ATP, since it cannot be reproduced by other chelating agents.

The affinity of ATP for the enzyme is lower than that of most true coenzymes, a concentration of 3–4 mM being required for full saturation. The activity of the deamination reaction is therefore variable within a concentration range of ATP corresponding to its physiological concentration range in animal tissues. If the concentration of ATP is low, the deamination of AMP will be slow and more AMP will remain available for rephosphorylation to ATP. A high ATP concentration will have the reverse effect. This is an example of a biochemical feed-back mechanism.

According to Lyubimova & Matlina (1954) the adenylic deaminase associated with myosin is also activated by ATP. It is possible therefore that the

Table 12. *Paper chromatography of reaction mixture before and after incubation with particulate fraction for 2 hr.*

| Sample | Position of spot | $E_{250\ m\mu}$ | $E_{260\ m\mu}$ | $E_{250\ m\mu}/E_{260\ m\mu}$ | µmoles/spot |
|---|---|---|---|---|---|
| Initial | ATP | 0·500 | 0·605 | 0·83 | 0·38 |
| | AMP | 0·590 | 0·695 | 0·85 | 0·44 |
| Final | ATP | 0·490 | 0·590 | 0·83 | 0·37 |
| | IMP | 0·525 | 0·337 | 1·56 | 0·43 |

Table 13. *Electrophoresis on paper of reaction mixture before and after incubation with particulate fraction for 2 hr.*

| Sample | Position of spot: Distance travelled (cm.*) | Position of spot: Reference compound, having the same rate of movement | $E_{250\ m\mu}$ | $E_{260\ m\mu}$ | $E_{250\ m\mu}/E_{260\ m\mu}$ | µmoles/spot |
|---|---|---|---|---|---|---|
| Initial | 20·5 | ATP | 0·645 | 0·755 | 0·85 | 0·48 |
| | 6·0 | AMP | 0·650 | 0·760 | 0·85 | 0·48 |
| Final | 20·0 | ATP | 0·605 | 0·710 | 0·85 | 0·45 |
| | 13·0 | IMP | 0·560 | 0·380 | 1·47 | 0·45 |

* Measured from centre of spot.

phenomenon is not restricted to the adenylic deaminase of brain.

The observation that the enzyme is associated with insoluble particles is interesting in view of the adenylic deaminase activity of brain mitochondria (Weil-Malherbe & Green, 1955). Abood & Gerard (1954) found that in peripheral nerve of the rat adenylic deaminase is mainly associated with small microsomes of less than 100 m$\mu$. in diameter.

## SUMMARY

1. Adenylic deaminase of ox brain was purified 10- to 20-fold by fractionating the aqueous extract of acetone powder with ammonium sulphate and alumina gel C$\gamma$.

2. The activity is associated with insoluble particles which can be separated on the high-speed centrifuge. Treatment with sodium cholate, though inhibitory, produces some solubilization, as shown by the appearance of activity in the supernatant solution.

3. Under standard conditions the preparation is practically free of adenosinetriphosphatase, 5-nucleotidase, myokinase and adenosine deaminase activities.

4. The activity of the enzyme is greatly increased by adenosine triphosphate. The effect is specific; adenosine diphosphate, inosine tri- and diphosphates are inactive. Some activity persists even in complete absence of adenosine triphosphate. This activity cannot be accounted for by adenosine deaminase action.

5. The effect of varying adenosine triphosphate concentrations on the activity of the enzyme is independent of the concentration of adenylic acid.

6. The pH optimum is 6·2–6·8.

7. The enzyme is inhibited by some multivalent cations and by phosphate and fluoride. Other inhibitors were tested.

8. Adenosine triphosphate is recovered unchanged at the end of the reaction, while adenylic acid is converted into inosinic acid and ammonia.

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# The Use of Paper Chromatography for *in vitro* Studies of Lactose Metabolism in Mammary-gland Preparations

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A major problem in the investigation of lactose synthesis by mammary-gland preparations is the estimation of lactose in the presence of other sugars, chiefly glucose. Previous workers (Grant, 1935, 1936; Knodt & Petersen, 1945; Malpress & Morrison, 1950; Hills & Stadie, 1952; Reithel, Horowitz, Davidson & Kittinger, 1952) have all used selective fermentation, combined with chemical methods more or less specific for the carbohydrates concerned. The development of paper partition chromatography of sugars (Partridge, 1948) has given the opportunity of carrying out a preliminary physical separation of lactose.

In the work described here quantitative paper chromatography was so adapted that lactose could be estimated in the contents of Warburg vessels in

which the gas exchange of various mammary-gland preparations had been measured. A synthesis of lactose obtained by Grant (1935) in guinea-pig mammary gland was confirmed, and some factors influencing it were further investigated. In addition, unsuccessful attempts were made to obtain lactose synthesis in cell-free preparations of the glands.

## MATERIALS

*Sugar phosphates.* Glucose 1-phosphate was prepared by the method of Hawk, Oser & Summerson (1947) and galactose 1-phosphate by the method of Colowick (1938). The method of preparation and the rotation of galactose 1-phosphate suggest that it is the α-isomer (Kosterlitz, 1939). The barium salt of glucose 6-phosphate and the calcium salt of fructose 1:6-diphosphate, both products of Schwarz Laboratories Inc., were converted into sodium salts in solution by the addition of $Na_2SO_4$ and sodium oxalate respectively.

*Purines.* Adenosine triphosphate (ATP) was prepared as the barium salt (LePage, 1949) and converted into the sodium salt (Eggleston & Hems, 1952). Inosine monophosphate (IMP) was a sample prepared by the method of Ostern (1932). Inosine was prepared by the method of Levene & Jacobs (1910).

Adenosine monophosphate (AMP) and hypoxanthine were the preparations of Roche Products, and adenosine the preparation of L. Light and Co. Ltd.

## METHODS

Lactating Norway rats (Wistar strain) or guinea pigs were killed and the mammary glands immediately removed and transferred to a dish of ice and water.

*Tissue slices.* These were cut with the tissue-slicer of Stadie & Riggs (1944) and about 100 mg. fresh weight of slices weighed and transferred to 4·0 ml. chilled medium in each of a series of Warburg flasks; the first slice was always discarded. In each experiment additional slices were dried at 110° for 16 hr. for measurement of dry weight.

The following salines were used. (1) The bicarbonate medium I, without substrates, of Krebs (1950); atmosphere, $O_2+CO_2$ (95:5), except in anaerobic experiments. (2) The calcium-free phosphate saline of Krebs & Eggleston (1940); 100% $O_2$. (3) The medium III without substrates, of Krebs (1950); 100% $O_2$. This medium contains low concentrations of phosphate and bicarbonate but the physiological concentration of calcium.

*Blends.* The mammary tissue of lactating guinea pigs was chopped into 40 ml. 0·9% (w/v) KCl at 0° and blended in the Waring Blendor for 90 sec. The pH was always found to be 7. Samples were added to chilled Warburg flasks containing the rest of the incubation mixture and other samples were dried. The dry weights were corrected for KCl. The atmosphere used was 100% $O_2$ or 100% $N_2$.

*Mitochondrial suspensions.* Method 1. The lactating guinea-pig mammary tissue was chopped into 40 ml. 0·25M sucrose in the Waring Blendor at 0° and blended for 90 sec. Mitochondrial suspensions in 0·9% KCl were then prepared by differential centrifugation, according to Bartley & Davies (1954).

Method 2. Blends made with 40 ml. 0·9% KCl (above) were similarly centrifuged. The supernatant of extra-mitochondrial cytoplasm in KCl from the first centrifuging was added to 5 ml. boiling 0·9% KCl and boiled for a further 10 min. The cooled solution was used as a suspending medium for the mitochondria, as a source of possible cofactors. The atmosphere was 100% $O_2$. The dry weights were corrected for KCl.

*Incubation.* The flasks were equilibrated in the Warburg bath at 40° for 10 min. before readings were taken. When 100% $O_2$ was used 0·2 ml. 2N-NaOH was placed in the centre well to absorb $CO_2$; in anaerobic experiments phosphorus was placed in the centre well.

*Inactivation.* Preliminary experiments showed that if rat-tissue slices were added to boiling water, dried at 110° for 16 hr. and then extracted with water, only 10% of the lactose was recovered. The procedure adopted was therefore to withdraw the control and incubated slices from the medium and to grind them with about 30 mg. $HgCl_2$ and a little sand. The incubation medium was added gradually during the grinding and the whole mixture centrifuged.

Blends and mitochondrial suspensions were deproteinized with 40% (w/v) trichloroacetic acid (TCA) (0·3 ml. for each 4·0 ml. of incubation mixture) and the mixtures centrifuged.

*Paper partition chromatography.* The apparatus and methods were essentially those of Bealing & Bacon (1953). The solvents were butanol–acetic acid–water (Partridge, 1948), *iso*butyric acid–ammonia (Krebs & Hems, 1953), and butanol–pyridine–water (6:4:3, by vol.). The first solvent, which was used for most purposes, does not separate AMP from IMP, or adenosine from hypoxanthine, and the second solvent was used for these separations. The third solvent was used to separate lactose from monosaccharide phosphates.

Sugars were detected with the benzidine–trichloroacetic acid spray of Bacon & Edelman (1951). Phosphates were detected according to Hanes & Isherwood (1949) and Bandurski & Axelrod (1951). Purines and their derivatives were detected as dark areas under u.v. light (Chromatolite Lamp, Hanovia Ltd., Slough, Bucks).

*Quantitative estimation of lactose.* Each supernatant was applied to the base line of a 24 × 24 in. sheet of Whatman no. 1 paper with an Agla micrometer syringe (Burroughs Wellcome Ltd.) in four groups of four 5 μl. spots, with five 5 μl. guide spots. The spots were dried with a hair drier (cold air in the case of TCA solutions) and the application was repeated twice more. Mixtures of 2% lactose, 0·5% galactose and 0·5% glucose (two 5 μl. spots) were used as guide spots on each chromatogram. The chromatograms were developed for 15 hr. or more in butanol–acetic acid–water. After drying and spraying the guide strips, the four corresponding lactose areas and similar blank areas in front of the monosaccharide position were cut from the chromatograms. The sugars were eluted by shaking with 8·0 ml. water in 6 in. glass-stoppered tubes and heating at 80° for 30 min. The paper was squeezed to the bottom of the tube and the solution filtered.

The filtrates were estimated by the reducing-sugar method of Nelson (1944) and Somogyi (1945). Two samples (2·0 ml.) were transferred to 6 in. glass-stoppered tubes (internal diam. 1 cm.), graduated at 8·0 ml. Two standard lactose solutions (20 μg. in 2·0 ml.) and a reagent blank (2·0 ml. water) were also taken. Throughout the present work the copper reagent of Somogyi (1945) was prepared

with half the concentration (i.e. 0·4%, w/v) of $CuSO_4,5H_2O$ in an attempt to reduce the blank. The resultant blank was paler, but so were the colours of the lactose determinations, so that the use of this modified reagent eventually proved to be of no advantage. The copper reagent (2·0 ml.) was added, the solutions were mixed and the tubes heated in a boiling-water bath for 15 min.; this time was found to be sufficient for complete reduction. After cooling in a water bath (5 min.), 1·0 ml. of arsenomolybdate reagent (Nelson, 1944) was added and the solutions were mixed. The blue-green solutions were allowed to stand (5 min.) to allow the colour to develop and were then made up to the 8·0 ml. mark.

The absorption spectrum obtained by reading the colour from 50 $\mu$g. lactose against a reagent blank in the Unicam spectrophotometer showed a single peak with a maximum of 760 m$\mu$. Fig. 1 shows a lactose concentration curve made by using the Spekker absorptiometer with a Chance OR2 (red) glass filter (50% transmission at 610 m$\mu$.; 94% transmission at 650 m$\mu$. and over) this procedure was used throughout.

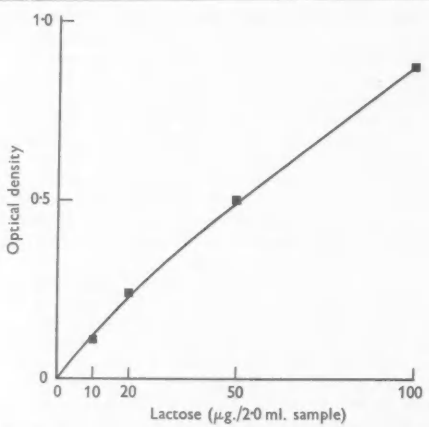


Fig. 1. Concentration curve of lactose estimation. Details of procedure given under 'Methods'. Each point represents the mean of four readings and twice the S.E.M.

The average of six paper blank readings was subtracted from the average reading for each lactose 'unknown', before comparison with the average reading for the lactose standard. The recovery of lactose from paper chromatograms was 98±1%.

*Statistical treatment.* Results are given in the form:

mean±S.E.M. (number of observations)
range of observations.

The test of significance applied to differences between means corresponds to a probability of 0·05.

## RESULTS

### *Slices from guinea-pig mammary gland*

Thirty-nine experiments were carried out with slices from lactating guinea-pig mammary glands; in twenty-six of these there was a significant synthesis of lactose. The results given below illustrate some aspects of the process.

*Time curve of lactose synthesis.* The results of an experiment in which slices were incubated for periods up to 3 hr. are plotted in Fig. 2. There was a significant synthesis of lactose in 2 hr. The mean $-Q_{O_2}$ values were 9·3 at 10–60 min., 8·1 at 60–120 min. and 7·5 at 120–170 min.

*Choice of saline.* The three experiments in which the syntheses in the different salines were compared are summarized in Table 1. No significant synthesis was obtained in the calcium-free, phosphate saline (2). There were significant syntheses in the bicarbonate saline (1) and in saline (3). No further

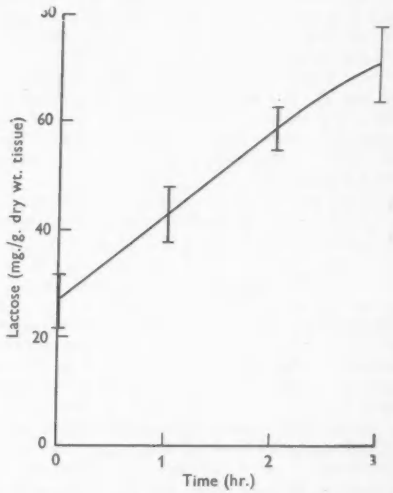


Fig. 2. Time curve of lactose synthesis in slices of lactating guinea-pig mammary gland (2 days). Saline (3) with the following substrates added: 15 mM glucose, 5 mM glutamate, 5 mM pyruvate and 6 mM fumarate. The vertical lines represent twice the S.E.M. of three observations.

investigation of calcium-free saline was made. In most subsequent experiments saline (3) was preferred, because $O_2$ uptake could be measured with it.

By applying the $-Q_{O_2}$ values in saline (3) to the gas exchange in saline (1) the R.Q. values were calculated to be 1·3 (Expt. 2) and 1·4 (Expt. 3). These agree with other observations on tissue slices of lactating non-ruminant mammary gland, e.g. 1·6 for rat (Folley & French, 1949).

*Distribution of lactose between tissue slice and medium.* In five early experiments the slices and medium were analysed separately. For example, Table 2 shows a typical experiment in which the

Table 1. *Effect of different salines on lactose synthesis in tissue slices*

Details of experiments as described under 'Methods'; in each case the saline contained 17 mM glucose. Each experiment was carried out with tissue from a single guinea pig; the time in parenthesis indicates the stage of lactation.

| Expt. | Time (hr.) | Saline | $-Q_{O_2}$ | Lactose content (mg./g. dry wt.) | Synthesis |
|---|---|---|---|---|---|
| 1 (3 days) | 0 | — | — | 17±3·0 (3) 14–23 | — |
| | 2 | 1 | — | 37±4·5 (3) 28–42 | Significant |
| | 2 | 2 | 9·7 | 21±3·9 (3) 14–27 | — |
| 2 (2 days) | 0 | — | — | 12±1·4 (3) 10–15 | — |
| | 2 | 1 | — | 28±3·0 (3) 24–34 | Significant |
| | 2 | 2 | 7·1 | 15±4·4 (3) 10–24 | — |
| | 2 | 3 | 7·2 | 21±4·9 (3) 12–29 | — |
| 3 (2 days) | 0 | — | — | 50±12·0 (3) 38–74 | — |
| | 3 | 1 | — | 96±6·6 (3) 83–105 | Significant |
| | 3 | 2 | 9·4 | 84±6·8 (3) 77–98 | — |
| | 3 | 3 | 9·1 | 121±23·6 (3) 84–165 | Significant |

Table 2. *Distribution of lactose between tissue slices and medium*

The incubation medium (saline 1) was applied directly to chromatograms for separation and estimation of lactose. The tissue slices were ground with sand and 1·0 ml. 10 mM-$HgCl_2$; the mixture was then centrifuged and the supernatants were applied to chromatograms for lactose estimation.

| Substrate | Incubation time (hr.) | Fresh tissue* (g.) | Lactose in tissue (mg./g.) | Vol. of medium* (ml.) | Lactose in medium (mg./ml.) | Total lactose* (mg./g. fresh wt.) |
|---|---|---|---|---|---|---|
| — | 0 | 0·216 | 6·0 | — | — | 6·0 |
| 17 mM Glucose | 2 | 0·189 | 2·0 | 12 | 0·27 | 19·3 |
| 17 mM Glucose +50 mM glutamate | 2 | 0·224 | 2·1 | 12 | 0·30 | 18·3 |

* Values for material from three Warburg vessels.

Table 3. *Effect of anaerobiosis on lactose synthesis*

Slices of a lactating guinea-pig mammary gland (2 days) were incubated in saline (1) under the conditions described.

| Substrate (17 mM) | Atmosphere | Incubation time (hr.) | Lactose content (mg./g. dry wt.) |
|---|---|---|---|
| Glucose | — | 0 | 39±3·0 (3) 35–45 |
| Glucose | $CO_2+N_2$ (5:95) | 2 | 31±2·7 (3) 28–36 |
| None | $CO_2+O_2$ (5:95) | 2 | 32±0·9 (3) 31–34 |
| Glucose | $CO_2+O_2$ (5:95) | 2 | 72±2·9 (3) 66–75 |

final concentration of lactose in the tissue slice (mg./g. fresh wt.) was about 7 times that in the medium (mg./ml.). This procedure was discarded in favour of the procedure described under 'Methods'. The latter has the advantage that control slices and incubated tissue have identical treatment, and it also reduces the time spent on each experiment.

*Essential conditions for synthesis.* There was a significant synthesis when slices were incubated in the presence of $O_2$ and added glucose, but no synthesis in the absence of $O_2$ or of glucose (Table 3). There is no significant difference between the lactose contents of the first three groups in this table; the possible disappearance of lactose during incubation without glucose was investigated further (see below). Fig. 3 shows the relation of lactose synthesis and of $O_2$ uptake to glucose concentration; the $-Q_{O_2}$ and the synthesis of lactose increased with glucose concentration over the range 2·5–17 mM.

In each of the remaining experiments reported in this section, a control incubation with glucose showed significant lactose synthesis; unless otherwise stated, the concentration of glucose was 2·5 mM.

*Effect of 2:4-dinitrophenol (DNP).* The effects of this inhibitor of oxidative phosphorylation (Lehninger, 1949) are given in Table 4. In Expt. 1, 0·02 mM DNP gave a greater $-Q_{O_2}$ and a greater synthesis; in Expt. 2, 0·02 mM DNP gave a greater $-Q_{O_2}$ but the same synthesis. In Expt. 1, 0·2 mM DNP did not change the $-Q_{O_2}$ further, but there was no synthesis; in Expt. 2, 0·2 mM DNP increased the $-Q_{O_2}$ further and again there was no

synthesis. Thus both concentrations increased $-Q_{O_2}$ and 0·2 mM DNP inhibited synthesis; all the differences referred to were significant.

*Effect of substrates other than glucose.* (*a*) No effect was found when glutamate (see Table 2), fumarate, or pyruvate was added to 17 mM glucose. In one experiment 2·5 mM glucose + 5 mM pyruvate + 2 mM fumarate gave the same synthesis as 5 mM glucose + 2 mM fumarate.

(*b*) No change occurred in the lactose concentration when tissue slices were incubated with 9 mM lactose as substrate. In this experiment the control, which showed synthesis, contained 17 mM glucose.

(*c*) Table 5 shows the effect on lactose synthesis of galactose as a substrate in addition to glucose. The $-Q_{O_2}$ was as great in 3 mM glucose as in 6 mM glucose, so that the amount of substrate available for respiration was not limiting the lactose synthesis. There was a significant synthesis with 3 mM glucose, and a significant increase on this synthesis with 6 mM glucose, so that the rate with 3 mM glucose was not already maximal. The synthesis in 3 mM glucose + 3 mM galactose was significantly less than the synthesis in 6 mM glucose.

(*d*) A significant synthesis of lactose occurred when tissue slices were incubated with 2·5 mM glucose 1-phosphate as substrate. Paper chromatography showed that about 90% of glucose 1-phosphate disappeared and glucose and inorganic phosphate appeared.

(*e*) A significant synthesis of lactose occurred in 2·5 mM glucose (Table 6), but the addition of

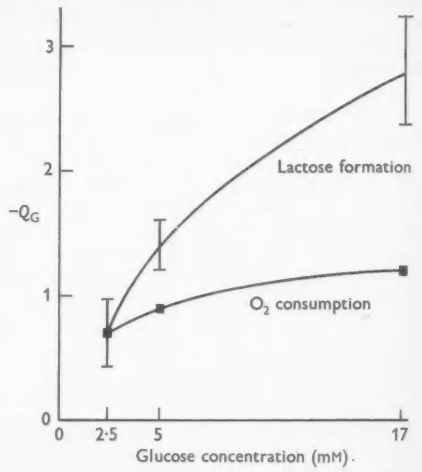


Fig. 3. The effect of glucose concentration on $O_2$ uptake and on lactose synthesis in tissue slices of lactating guinea-pig mammary gland. The curves are based on seven experiments in which there was a significant synthesis with saline (3) and with glucose as sole substrate. $-Q_G$ (lactose) and $-Q_G$ ($O_2$) are $\mu$l. glucose used/mg. dry wt. of tissue/hr. calculated from the lactose synthesized and from $O_2$ uptake, respectively, on the assumptions that glucose is the sole substrate for synthesis and for oxidation. The vertical lines indicate the S.E.M. of the mean values from the separate experiments. The squares include the S.E.M. for $-Q_G$ ($O_2$).

Table 4. *Effect of 2:4-dinitrophenol (DNP)*

In each experiment mammary-gland slices from a lactating guinea pig (3 days) were incubated in saline (3) plus 5 mM glucose, with or without DNP. The zero-hour samples included one from each DNP concentration.

| Expt. | DNP (mM) | Incubation time (hr.) | $-Q_{O_2}$ | Lactose content (mg./g. dry wt.) |
|---|---|---|---|---|
| 1 | — | 0 | — | 33±3·2 (3) 27–38 |
| | 0 | 2 | 5·0±0·1 | 47±1·2 (3) 45–49 |
| | 0·02 | 2 | 7·5±0·3 | 56±2·8 (3) 53–62 |
| | 0·20 | 2 | 6·6±0·5 | 38±1·7 (3) 35–41 |
| 2 | — | 0 | — | 26±0·9 (3) 25–28 |
| | 0 | 2 | 7·3±0·4 | 52±2·3 (3) 48–56 |
| | 0·02 | 2 | 9·5±0·1 | 49±4·4 (3) 42–57 |
| | 0·20 | 2 | 10·2±0·1 | 34±3·4 (3) 28–39 |

Table 5. *Effect of galactose as an additional substrate*

Mammary-gland slices from a lactating guinea pig (3 days) were incubated in saline (3) plus mM glutamate, 6 mM fumarate and 5 mM pyruvate, with the additions of monosaccharide specified.

| Further substrates (3 mM) | Incubation time (hr.) | Average $-Q_{O_2}$ | Lactose content (mg./g. dry wt.) |
|---|---|---|---|
| Glucose + galactose | 0 | — | 18±2·6 (3) 13–22 |
| Glucose | 2 | 7·3 | 29±1·2 (3) 27–31 |
| Glucose + galactose | 2 | 7·3 | 35±1·6 (3) 31–37 |
| Glucose (6 mM) | 2 | 7·4 | 47±1·7 (3) 44–50 |

Table 6. *Glucose plus α-galactose 1-phosphate as substrates for lactose synthesis*

Mammary-gland slices from a lactating guinea pig (3 days) were incubated in saline (3). The zero-hour samples included one of each substrate type.

| Substrates (2·5 mM) | Incubation time (hr.) | $-Q_{O_2}$ | Lactose content (mg./g. dry wt.) |
|---|---|---|---|
| — | 0 | — | 39±3·3 (3) 32–42 |
| Glucose | 2 | 4·6 | 51±1·2 (3) 49–53 |
| Glucose + galactose 1-phosphate | 2 | 4·7 | 55±3·6 (3) 44–57 |
| Glucose (5 mM) | 2 | 4·7 | 66±6·3 (3) 53–73 |

2·5 mM galactose 1-phosphate gave no greater synthesis. Chromatography showed the formation of free galactose though much galactose 1-phosphate remained.

*Dry/fresh weight quotient.* In each experiment there was close agreement between duplicates and the result from all experiments was: 0·24 ± 0·004 (72) 0·19–0·35.

*Slices of rat mammary gland*

There was much variation in the dry/fresh weight quotients, even between duplicate determinations in individual experiments, and the result from all experiments was 0·34 ± 0·019 (18) 0·21–0·47.

No synthesis of lactose was gained when slices of glands from lactating rats (7–17 days) were incubated under the same conditions as guinea-pig slices, e.g. in saline (1) with 17 mM glucose as substrate. There was always a net output of gas, indicating an R.Q. greater than 1. Incubation periods up to 6 hr.; the addition of 5 mM glutamate + 5 mM fumarate; the addition of 0·1 % (w/v) Pronutrin (Atomised Food Products and Herts Pharmaceuticals Ltd.) + 0·005 % (w/v) tyrosine, as a source of amino acids; and the addition of 0·25 % (w/v) 'Anterior lobe of pituitary gland substance' (Armour Laboratories, Chicago) did not produce a synthesis of lactose. In a typical experiment, in which 0·1 % (w/v) Pronutrin, 0·005 % (w/v) tyrosine and 5 % (fresh w/v) of a rat-liver extract were added, the lactose content (mg./g. dry wt.) was 13·2 at 10 min. and remained at 13·5 after 20, 40 and 60 min. incubation.

*Blends*

A blend (264 mg. dry wt./4·0 ml.), prepared from the glands of a lactating guinea pig (3 days), was incubated with 10 mM glucose, 5 mM ATP, 5 mM-$MgCl_2$ and 10 mM phosphate buffer (pH 7·4) in $O_2$ or $N_2$ for 20 or 60 min. The $-Q_{O_2}$ fell from 7·2 (10–20 min.) to 1·2 (50–60 min.). Some adenosine diphosphate (ADP) remained after 20 min. aerobic incubation, but in all other samples no ATP or ADP was detectable by paper chromatography. Little glucose disappeared; there was no synthesis of lactose.

Similarly, there was no change in the lactose present when blends were incubated for up to 1 hr. in $O_2$ with 12 mM α-oxoglutarate, mM ATP, mM-$MgCl_2$, 10 mM phosphate buffer (pH 7·4), and either 5 mM glucose or 5 mM lactose.

Paper chromatography showed that during the incubations with blends ATP was broken down and AMP, inosine, and hypoxanthine appeared. No adenosine was detected, but the incubation of blends with adenosine gave rise to inosine and hypoxanthine, so that it is not excluded that adenosine was an intermediate.

*Mitochondrial suspensions*

In the experiment described in Table 7 the $-Q_{O_2}$ decreased rapidly in the presence of succinate or α-oxoglutarate, but was greater and continued with only a small decrease in the presence of pyruvate. Fumarate was added in catalytic amounts to aid the oxidation of pyruvate by the tricarboxylic acid cycle, but it appeared from the incubations with succinate and α-oxoglutarate that oxidation was not proceeding beyond fumarate. Chromatograms showed that ATP concentration was highest in the presence of pyruvate and fumarate, but had nevertheless fallen from its original level. No lactose was formed.

Table 7. *Incubation of mitochondria with various substrates*

The mitochondrial suspension was prepared by method 1 from the mammary glands of a lactating guinea pig (4 days). Each incubation mixture (4·0 ml.) contained 113 mg. dry wt. mitochondria, 5 mM glucose, 2 mM ATP, mM-$MgCl_2$ and 10 mM phosphate buffer (pH 7·4).

| Additional substrates (5 mM) | $-Q_{O_2}$ 10–20 min. | $-Q_{O_2}$ 20–30 min. |
|---|---|---|
| Succinate | 10 | 1 |
| α-Oxoglutarate | 16 | 5 |
| Pyruvate + fumarate (0·5 mM) | 23 | 21 |

In incubations of mitochondrial suspensions (method 2) in the presence of 10 mM pyruvate and mM fumarate, there was no change in the lactose

concentration or in the appearance of butanol–acetic acid or butanol–pyridine chromatograms with the following additional substrates: glucose + galactose, glucose + glucose 1-phosphate; glucose + glucose 6-phosphate; glucose + hexosediphosphate; glucose + glucose 1-phosphate + glucose 6-phosphate + galactose 1-phosphate (all at 2·5 mM); nor with glucose, galactose, or glucose 1-phosphate (all at 5 mM).

*Mammary-gland lactase*

Extracts of lactating guinea-pig or rat mammary gland were prepared by grinding with sand. When these extracts were incubated in stoppered tubes with 4% (w/v) lactose at 37° in the presence of $CHCl_3$, for 24 hr. there was a formation of glucose and galactose and a slight formation of oligosaccharides. Extracts prepared in a stainless-steel homogenizer of the Potter & Elvehjem (1936) type, and acetone powder extracts, had similar activity, but preparations made in the Waring Blendor were more active. The lactase activity was strongest at pH 4–5 and was unaffected by dialysis of the extracts or by the addition of inorganic phosphate or fluoride. No phosphate corresponded with the sugar positions on butanol–acetic acid chromatograms and there was no correlation of the lactase activity with acid phosphatase; one preparation was obtained with no acid phosphatase activity. In a typical experiment, in which an extract made with sand was incubated with added lactose for 22 hr. at pH 5 with 0·2 M acetate buffer and 0·02 M-$KH_2PO_4$ at 37°, there was a breakdown of lactose from 2·1 to 1·8%, and a corresponding increase in monosaccharide.

## DISCUSSION

Despite the observations of Malpress & Morrison (1949), paper chromatography has proved to be a convenient method of following lactose synthesis in tissue slices. It has the advantage that the nature of the sugar being estimated can be confirmed, and usually it is possible to decide by inspection of the chromatograms whether synthesis has taken place. Any unusual carbohydrate in the extract may also be discovered (cf. Heyworth & Bacon, 1954). Even when relatively large amounts of glucose are present a preliminary fermentation with yeast is not needed; this avoids some of the complications, e.g. production of dextrins, introduced by the use of yeast.

In the present work lactose has been estimated with smaller amounts of tissue and shorter times of incubation than those used by previous workers: 25 mg. dry wt. and 2 hr. respectively [cf. 80 mg., 6 hr. (Grant, 1935); 450 mg., 4 hr. (Malpress & Morrison, 1950)]. The rates of lactose synthesis measured with slices of guinea-pig gland were somewhat higher than those recorded previously; an average of 18 mg./g. dry wt./hr. was found with 17 mM glucose as substrate. Grant (1935) found about 8 mg., and Malpress & Morrison (1950), 3 mg. The rate was dependent upon glucose concentration within the physiological range.

The failure to demonstrate synthesis with guinea-pig tissue in calcium-free phosphate saline may have some connexion with the calcium-secreting activity of the mammary gland. The possibility that lactose secretion in slices was linked to synthesis of other milk constituents was also considered when experiments with rat tissue failed to show signs of synthesis from glucose alone; however, the addition of an amino acid mixture had no effect. We can offer no explanation of the failure to demonstrate synthesis in rat tissue slices. It would seem unlikely that the lactose level in these experiments represented a balance of synthesis and breakdown, because it remained always remarkably constant, and no breakdown of lactose in the absence of added glucose was ever observed. Certain precautions were found to be necessary in measuring the total lactose content of an incubation system. A falsely low value of the lactose content of slices may be obtained if they are dried and afterwards extracted. As Table 2 shows, lactose may be lost from slices to medium, so that the lactose content of the slices may fall even when a net synthesis of lactose is taking place. Judged by these criteria, the experimental details given by Hills & Stadie (1952), who found a synthesis of about 5 mg. lactose/g. dry wt./hr. in rat tissue slices, are insufficient.

Attempts to elucidate the mechanism of lactose synthesis with tissue slices were for the greater part unsuccessful. The absence of synthesis under anaerobic conditions (cf. Malpress, 1950) and with 2:4-dinitrophenol indicates that it is dependent upon oxidative phosphorylation. [This conclusion must be qualified by a reference to the rates of synthesis which can be measured by our method. Reithel *et al.* (1952) and Kittinger & Reithel (1954), who obtained a synthesis under anaerobic conditions, have used radioactive substrates as a means of measuring rates of synthesis too small to be measured by our method.]

The possibility that free galactose is an intermediate in the synthesis of lactose from glucose was tested by incubations with mixtures of glucose and galactose. The fact that galactose was not as effective as glucose in supplementing a suboptimum concentration of glucose may be taken as evidence against this possibility, but only if it is assumed that both sugars are absorbed by slices at an adequate rate.

Although lactose is a $\beta$-galactoside, the discovery that maltose, an $\alpha$-glucoside, may be phosphorolysed to yield $\beta$-glucose 1-phosphate (Fitting & Doudoroff, 1952) makes it conceivable that

α-galactose 1-phosphate could be a precursor of lactose; McGeown & Malpress (1952) found evidence for its presence in cow's milk. This substance is formed when galactose is phosphorylated by galactokinase, and also by the action of galactowaldenase (see Rutter & Hansen, 1953). When tested as a supplement to suboptimum glucose, it gave no significant increase in lactose synthesis. This again might be the result of a failure to reach the site of synthesis, either because of the existence of physical barriers, or because the substance is hydrolysed rapidly after gaining entry.

Many of the uncertainties mentioned above would be eliminated if a cell-free preparation could be obtained showing comparable rates of lactose synthesis. All our attempts to obtain such a preparation were unsuccessful. Reithel *et al.* (1952) found a variable but definite synthesis in cell-free preparations by a colorimetric method, but later resorted to the use of isotopes to measure the very small rates of synthesis achieved with their partially purified extracts (about 0·1 mg./g. dry wt./hr.). It is possible that the preparations of Terner (1954), which respire more actively, might be capable of a higher rate of synthesis.

Preparations of mitochondria, which broke down ATP much less rapidly than unfractionated blends, also showed no synthesis. Although some synthetic reactions, e.g. citrulline synthesis (Müller & Leuthardt, 1949) and *p*-aminohippuric acid synthesis (Kielley & Schneider, 1950), are carried out by mitochondrial preparations, it would seem less likely that lactose synthesis would be found in such a preparation than in crude homogenates. Like the cyclophorase preparations of Moore & Nelson (1952), our mitochondrial preparations from mammary gland did not apparently oxidize succinate and α-oxoglutarate beyond the stage of fumarate. However, unlike theirs, our preparations would oxidize pyruvate beyond citrate without the addition of nucleotides.

We have obtained no evidence for the existence of a lactose phosphorylase, but there was weak lactase activity, with an optimum at pH 5. There was no detectable breakdown of lactose by tissue slices under more physiological conditions. The appearance of oligosaccharide with an $R_F$ value less than that of lactose suggests that the action of mammary-gland lactase, like that of the lactases of certain micro-organisms, involves transgalactosylation (Wallenfels & Bernt, 1952; Roberts & McFarren, 1953; Aronson, 1952; Pazur, 1953).

## SUMMARY

1. Various preparations from lactating mammary glands of guinea pigs and rats were used to study the metabolism of lactose *in vitro*.

2. The formation of lactose and the fate of substrates added to the preparations were followed by paper chromatography. A chromatographic method was developed by which rates of synthesis of lactose could be measured after incubation of tissue slices in Warburg manometer vessels.

3. Lactose was not broken down by respiring tissue slices, blends or mitochondrial suspensions, but crude aqueous extracts showed some lactase activity, particularly when the pH was lowered.

4. The synthesis of lactose obtained by Grant (1935) in surviving mammary tissue slices from guinea pigs was confirmed, but no synthesis was obtained with rat tissue. The synthesis was supported by glucose and showed a greater proportional increase with glucose concentration than did glucose oxidation. It was not increased by the partial replacement of glucose by other substrates. The synthesis was dependent on aerobic conditions and was inhibited by 0·2 mM DNP.

5. No synthesis of lactose was gained with blends or with mitochondrial suspensions prepared from guinea-pig tissue.

6. Succinate and α-oxoglutarate were apparently not oxidized beyond fumarate by mitochondrial suspensions from guinea-pig mammary gland.

We wish to thank Professor H. A. Krebs, F.R.S., and other members of the Medical Research Council Unit for Research in Cell Metabolism for their help in many ways; in particular, Mr D. H. Williamson for preparing glucose 1-phosphate, galactose 1-phosphate and inosine. One of us (R.H.) is indebted to the Medical Research Council for the award of a scholarship.

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# The Substrate Specificity and Inhibition of Alkaline Phosphatases of Cow's Milk and Calf Intestinal Mucosa

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It is somewhat surprising, in view of the extensive literature relating to alkaline phosphatases, that the substrate specificity of these enzymes has not been clearly established. Although it has been shown that they hydrolyse a number of different phosphate esters (Folley & Kay, 1936; Roche & Thoai, 1950), this information is derived from studies with crude or partially purified preparations. The widely accepted belief that hydrolysis is limited to orthophosphate monoesters (Roche & Thoai, 1950; Roche, 1950) is not well-founded, especially since Schmidt & Thannhauser (1943) showed that highly purified preparations from calf intestinal mucosa hydrolysed phosphate diesters and pyrophosphates as well as phosphate monoesters.

The substrate specificity has now been determined using purified phosphatases from cow's milk (Morton, 1950, 1953*a*) and calf intestinal mucosa (Morton, 1950, 1954). It has been found that only orthophosphate monoesters and closely related compounds, but not diesters or pyrophosphates, are hydrolysed.

Since these alkaline phosphatases may therefore be regarded as monoesterases of a special type, it seemed possible that they might be inhibited by compounds which inhibit other esterases. The failure to obtain appreciable inhibition by a range of organic pyrophosphates, phosphites and phosphonates shows the marked difference between these alkaline phosphatases and certain other esterases.

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## MATERIALS AND METHODS

### *Enzymes and buffers*

Unless otherwise indicated, the experiments described here were carried out in 1950 and early 1951 using alkaline phosphatases purified from cow's milk (Morton, 1950, 1953*a*) and from calf intestinal mucosa (Morton, 1950, 1954). The method of handling these preparations and of purifying and preparing the buffers used in this work is described in another paper (Morton, 1955*a*).

### *Substrates*

Except where otherwise indicated, commercial preparations were used. They were generally examined for the presence of contaminant phosphorylated compounds (Morton, 1955*a*), and, unless otherwise mentioned, were used without further purification.

*Phosphocreatine* (PC). The crystalline sodium salt was prepared by the method of Ennor & Stocken (1948).

*Phosphoenolpyruvate* (PEP). A preparation of the barium salt, which had been kept in the dry state for some years, was kindly supplied by Dr M. Dixon. It was purified by converting into the sodium salt, precipitating with barium acetate as described by LePage (1949) and subsequently removing inorganic phosphate and pyruvic acid according to Meyerhof & Oesper (1949). All the organic phosphate was iodine-labile when tested as described by Meyerhof & Oesper (1949).

*Adenosine triphosphate* (ATP). The dibarium salt was prepared from rabbit muscle by the late Mr E. J. Morgan, using the procedure of Lohmann as described by Needham (1942). The preparation also contained some adenosine diphosphate (ADP) and adenosine 5′-monophosphate (AMP).

*Adenosine diphosphate.* This was prepared by the late Mr E. J. Morgan by the action of rabbit muscle myosin-adenosine triphosphatase on ATP. The preparation also contained some ATP and AMP.

*Glucose 1-phosphate* (G 1-P). The crystalline potassium salt was kindly supplied by Dr D. M. Needham.

*Triose phosphate.* A mixture of glyceraldehyde phosphate and dihydroxyacetone phosphate, prepared according to the method of Meyerhof (1938) by the action of aldolase on hexose diphosphate, was kindly supplied by Dr D. M. Needham.

*Fructose 1-phosphate.* This was prepared synthetically by Dr G. Hers and kindly supplied by him.

*2-Phosphoglycerate, 3-phosphoglycerate and diphenyl pyrophosphate.* These were gifts from Dr M. Dixon.

*Diphosphopyridine nucleotide* (DPN). A preparation assaying 33% DPN was prepared by the late Mr E. J. Morgan and kindly supplied by him.

### *Other compounds*

Unless otherwise indicated below, the compounds investigated as potential inhibitors of alkaline phosphatases were commercial preparations.

Trimethyl phosphate and triphenyl phosphate were kindly supplied by Dr M. Dixon. Tetraethyl pyrophosphate, hexaethyl tetraphosphate and di*iso*propyl fluorophosphonate (DFP) were gifts from Dr E. C. Webb. Ammonium fluorophosphonate, diethyl phosphonate, tribenzyl borate and the various organic phosphites were gifts from Dr B. C. Saunders, Department of Organic Chemistry, University of Cambridge.

### *Methods*

*Hydrolysis of substrates.* The reaction was generally commenced by addition of 0·2 ml. of a suitable dilution of enzyme to 4·8 ml. of reaction mixture containing the substrate, magnesium acetate (0·01 M) and either ethanolamine–HCl (0·04 M) or veronal–HCl (0·04 M) buffer. All activities were determined at 38°. Generally, the reaction period was 5 min. but was extended as necessary. Unless otherwise indicated, hydrolysis was measured by estimation of the inorganic phosphate liberated enzymically. Hydrolysis of phenyl phosphate and of PC was usually measured by estimation of liberated phenol and liberated creatine, respectively. All pH values are given for 38°.

*Phosphate.* The method of Fiske & Subbarow (1925) was generally used. However, where acid-labile materials (PC and all compounds containing pyrophosphate linkages) were present in the reaction mixture, the method of Weil-Malherbe & Green (1951) was used.

*Acid-labile phosphate.* This was determined by estimation of inorganic phosphate liberated in 10 min. at 100° in 1 N-HCl.

*Creatine.* The colorimetric method of Eggleton, Elsden & Gough (1943) was used.

*Phenol.* The following modification of the colorimetric method of King (1951) was used. The enzymic reaction (total volume, 5 ml.) was stopped by the direct addition of 1·5 ml. of diluted (1/3) phenol reagent. Then 1 ml. of 25% $Na_2CO_3$ was added, the volume made up to 10 ml. with distilled water and the colour developed at 38° for 20 min.

*Inhibition of phosphatase activity.* Activities were determined as follows. The diluted enzyme, in veronal–sodium acetate–HCl buffer (0·02 M, pH 7·8) was treated with the inhibitor, and held at room temperature (about 18°) for 20 min. The tube was then brought to 38° and the reaction commenced by the addition of the buffered substrate–magnesium acetate mixture. Volumes were adjusted so that the concentration of the inhibitor during the activity test was half that during prior treatment of the enzyme. Final concentrations of the reactants, in a final volume of 1·0 ml., were as follows: phenyl phosphate, 0·02 M; magnesium acetate, 0·01 M; ethanolamine–HCl buffer (pH 9·95), 0·04 M. The reaction period was 5 min. Activity was estimated by determination of liberated phenol, except where interfering substances, such as cysteine, were present. In these cases, liberated inorganic phosphate was measured.

## RESULTS

### *Substrate specificities of purified phosphatases*

In order to determine the types of substrates which are hydrolysed by the purified milk and intestinal phosphatases, representative phosphorylated compounds were examined at selected substrate concentrations. As far as possible, each substrate was examined over a range of pH values chosen to include the optimum pH for the substrate concentration used. The results are shown in Table 1. In some cases a number of substrate concentrations were used, and the apparent $K_m$ (Michaelis's constant) value determined from a Lineweaver & Burk (1934) plot as modified by Dixon (1953*a*). These are shown in Table 2.

All orthophosphate monoesters examined, as shown in Table 1, were found to be rapidly hydrolysed by both purified phosphatases. Especial interest attaches to the results obtained with compounds other than true orthophosphates, and these are briefly considered in greater detail.

*Phosphoenolpyruvate.* The rapid hydrolysis of this compound by both enzymes confirms previous findings by Meyerhof & Green (1950), using partially purified phosphatase from calf intestinal mucosa.

*Phosphoamides.* Phosphocreatine was chosen as representative of the known biological compounds of this class. Because of the considerable lability of PC under acid conditions, the experiment was

carried out as follows. The enzymic reaction was commenced by the addition of enzyme (0·1 ml.) and stopped by addition of 0·2 ml. of 20% trichloroacetic acid (TCA) to the reaction mixture (2 ml.). After 15 sec., the mixture was brought to pH 8·0 by addition of the appropriate amount of 0·2 N-NaOH, and the liberated creatine or inorganic phosphate determined as indicated under Methods. Both phosphatases rapidly hydrolysed PC, and with both enzymes the rate of hydrolysis was markedly dependent on substrate concentration, increasing up to the maximum (0·05 M) used. The activity/pH curve for hydrolysis of PC (0·015 M) is compared with those for hydrolysis of β-glycerophosphate (0·02 M) and phenyl phosphate (0·025 M) in Fig. 1. It is seen that the pH has somewhat less influence on the rate of hydrolysis of PC by intestinal phosphatase than on the rate of hydrolysis

Table 1. *Compounds examined as substrates for purified milk and intestinal phosphatases*

Each compound, at the concentration indicated, was examined as described under Methods, either at an arbitrarily chosen pH value or over a range of pH values from about 8·2 to 10·2.

| | Compound tested | Concentration (M) | pH of reaction: Intestinal phosphatase | pH of reaction: Milk phosphatase |
|---|---|---|---|---|
| | **Hydrolysed** | | | |
| Orthophosphate monoesters and amides | Phenyl phosphate | $2·5 \times 10^{-3}$ | 9·8* | 10·05* |
| | *p*-Nitrophenyl phosphate | $1·0 \times 10^{-3}$ | 9·6 | 9·6 |
| | Phenolphthalein phosphate | $1·0 \times 10^{-3}$ | 9·6 | 9·6 |
| | α-Glycerol phosphate | $2·0 \times 10^{-2}$ | 9·3* | — |
| | β-Glycerol phosphate | $2·0 \times 10^{-2}$ | 9·45* | 9·65* |
| | 2-Phosphoglycerate | Approx. $1 \times 10^{-4}$ | 9·0 | — |
| | 3-Phosphoglycerate | Approx. $1 \times 10^{-4}$ | 9·0 | — |
| | Triose phosphate | Approx. $4 \times 10^{-4}$ | 9·0 | — |
| | Glucose 1-phosphate | $1·7 \times 10^{-3}$ | 9·3* | 9·5 |
| | Glucose 6-phosphate | $1·3 \times 10^{-3}$ | 9·2* | 9·5 |
| | Fructose 1-phosphate | Approx. $2 \times 10^{-4}$ | 9·45 | — |
| | Fructose 6-phosphate | Approx. $5 \times 10^{-4}$ | 9·45 | — |
| | Fructose 1:6-diphosphate | $1·2 \times 10^{-3}$ | 9·15 | 9·5 |
| | Adenosine 3′-phosphate | $5·2 \times 10^{-4}$ | 9·45* | 9·5 |
| | Adenosine 5′-phosphate | $4·5 \times 10^{-4}$ | 9·45* | 9·5 |
| | PC | $1·5 \times 10^{-2}$ | 9·55* | 9·55* |
| | PEP | $1·0 \times 10^{-2}$ | 9·65 | 9·5 |
| | **Not hydrolysed** | | | |
| Orthophosphate diesters | YNA | † | — | — |
| | Diphenyl phosphate | Approx. $1 \times 10^{-4}$ | — | — |
| Orthophosphate triesters | Trimethyl phosphate | Approx. $1 \times 10^{-2}$ | — | — |
| | Triphenyl phosphate | Approx. $1 \times 10^{-2}$ | — | — |
| Polyphosphates | Sodium pyrophosphate | $5·0 \times 10^{-4}$ | — | — |
| | Sodium hexametaphosphate | Approx. $1 \times 10^{-5}$ | — | — |
| | Diphenyl pyrophosphate | Approx. $1 \times 10^{-4}$ | — | — |
| | ATP | Approx. $6 \times 10^{-4}$ | — | — |
| | ADP | Approx. $5 \times 10^{-4}$ | — | — |
| | DPN | Approx. $6 \times 10^{-4}$ | — | — |

* Optimum pH values for hydrolysis at the substrate concentration shown.
† 120 μg. organic phosphate per 2 ml. final volume.

Table 2. *$K_m$ values for some substrates of alkaline phosphatases*

$K_m$ values were estimated graphically according to Lineweaver & Burk (1934) as modified by Dixon (1953*a*).

| Substrate | pH | $K_m$ value (M): Intestinal phosphatase | $K_m$ value (M): Milk phosphatase |
|---|---|---|---|
| Phenyl phosphate | 10·0 | $8·6 \times 10^{-4}$ | $6·1 \times 10^{-4}$ |
| β-Glycerol phosphate | 9·0 | $1·7 \times 10^{-2}$ | — |
| Adenosine 5′-phosphate | 9·45 | $1·1 \times 10^{-2}$ | — |
| Adenosine 3′-phosphate | 9·45 | $2·0 \times 10^{-4}$ | — |
| Phosphocreatine | 9·55 | $3·5 \times 10^{-2}$ | $3·5 \times 10^{-2}$ |

of other typical orthophosphate monoesters when examined at approximately the same substrate concentration.

The $K_m$ values at pH 9·6, obtained with milk and intestinal phosphatases, are considerably greater than those obtained for other substrates examined (Table 2), indicating a rather low affinity of these phosphatases for PC.

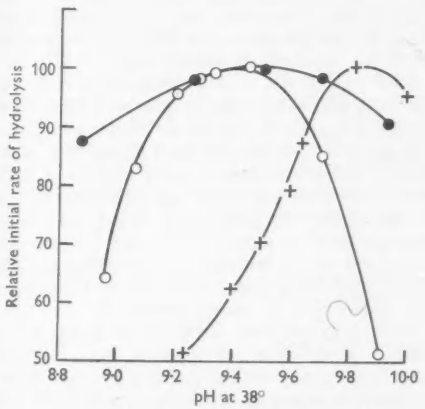


Fig. 1. Activity/pH curves for hydrolysis of phosphocreatine (PC), β-glycerophosphate and phenyl phosphate by purified intestinal alkaline phosphatase. The reaction mixtures contained magnesium acetate (0·01 M), ethanolamine–HCl buffer (0·04 M) and substrates at the concentrations shown. The reaction period was 5 min. at 38°. Hydrolysis was estimated as described under Methods. Results are expressed as μg. P liberated in 5 min. by the quantity of enzyme indicated. ●—●, PC (0·015 M), with approx. 12 μg. enzyme; ○—○, β-glycerophosphate (0·02 M), with approx. 2·1 μg. enzyme; +—+, phenyl phosphate (0·025 M), with approx. 1 μg. enzyme.

It is noteworthy that PC was hydrolysed by a partially purified acid phosphatase from human prostate gland (Morton, 1952). At pH 5·5, the initial rate of hydrolysis with 0·005 M PC was 102 μg. and with 0·05 M PC, 20 μg. P/min./0·1 ml. enzyme. High PC concentration thus causes marked inhibition of hydrolysis of PC by this enzyme, in marked contrast to the results obtained with the alkaline phosphatases.

*Orthophosphate diesters.* A commercial preparation of partially degraded yeast nucleic acid (YNA) was used as a source of naturally occurring phosphate diesters. The preparation was found to contain some free purines, adenylic acids *a* and *b*, and polynucleotides.

With a large amount of enzyme (approx. 20 μg. purified intestinal phosphatase) at pH 9·3, approx. 30 % of the total organic phosphate (120 μg.) was liberated in 90 min., and 40 % after 18 hr. The experiment was repeated by adding crystalline ribonuclease (approx. 2 μg.) before the addition of phosphatase. The initial rate of hydrolysis was not greatly increased (45 % of the total phosphate liberated in 90 min.), but 80 % of the total phosphate was liberated after 18 hr.

These results, obtained early in 1950, may now be explained in terms of the structure of YNA, which has since been elucidated (see Davidson, 1953). The intestinal phosphatase rapidly removed the monoester groups of the mononucleotides, larger polynucleotides and YNA. In the preparation of YNA used, this accounted for some 40 % of the total organic phosphate. The failure of the purified phosphatase to liberate any further inorganic phosphate, even after prolonged incubation, indicates the absence of diesterase activity.

It is clear from the results obtained in the presence of added ribonuclease, a specific phosphate diesterase, that diesterase action, together with phosphatase, would liberate up to 80 % of the total organic phosphate of the YNA. The failure to hydrolyse all the organic phosphate links is due to the ribonuclease-resistant 'core' of YNA.

The absence of diesterase activity from the intestinal phosphatase preparation has been further confirmed by Dr R. H. Markham (personal communication), using a phosphate diester of guanosine and uridine as substrate. Likewise diphenyl phosphate was not hydrolysed by intestinal phosphatase.

*Pyrophosphates.* Inorganic (0·0005 M) and diphenyl (0·0001 M) pyrophosphates were each examined, over the pH range 7·5–10·2. There was no enzymic hydrolysis by either milk or intestinal phosphatase after 90 min. or after 18 hr. incubation, using 10 μg. of purified enzyme in 3 ml. reaction mixture.

In testing the action of the purified phosphatases on ATP and ADP, approx. 10 μg. of protein was added to the reaction mixture (3 ml.) over the pH range 8·4–10·2. Inorganic phosphate was estimated after 15 min. With both enzymes there was maximum liberation of inorganic phosphate from the ATP preparation at pH 8·8. The course of the reaction was therefore followed at pH 8·8. As shown by the results in Fig. 2, there was an initial rapid reaction during the first 15 min., after which little further inorganic phosphate appeared. Approx. 5 % of the total organic phosphate was liberated after 90 min. However, after 17 hr. at pH 8·8, approx. 19 % of the total organic phosphate had appeared as inorganic phosphate. Similar results were obtained with both enzymes.

The results indicate that ATP is not hydrolysed by either phosphatase. The initial rapid liberation

of inorganic phosphate is attributed to the hydrolysis of AMP, shown by chromatography to be present. Moreover, by comparison of the acid-labile phosphorus (197 $\mu$g.) with total organic phosphorus (320 $\mu$g.) it was estimated that the preparation contained between 5 and 10 % AMP. The liberation of 19 % of the organic phosphate, following prolonged incubation (17 hr.) appears to be due to the non-enzymic splitting of ATP in alkaline solution, giving inorganic pyrophosphate and AMP, the latter being hydrolysed by intestinal phosphatase. Since essentially the same amount of inorganic phosphate was liberated with twice the amount of enzyme (Fig. 2), it cannot be attributed to enzymic hydrolysis of ATP.

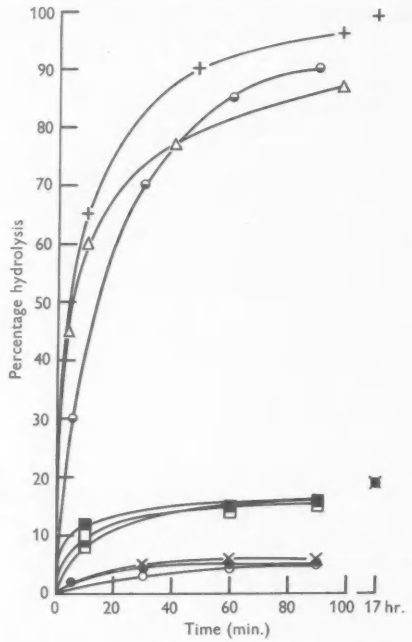


Fig. 2. The action of purified alkaline phosphatases on ATP, ADP, AMP and PC. The reaction mixture (3 ml.) contained ethanolamine–HCl buffer (0·04 M), magnesium acetate (0·01 M) and the amount of organic phosphorus shown.

ATP (320 $\mu$g. P) at pH 8·8: ○—○, with milk phosphatase (approx. 10 $\mu$g.); ●—●, with intestinal phosphatase (approx. 10 $\mu$g.); ×—×, with intestinal phosphatase (approx. 20 $\mu$g.).

ADP (185 $\mu$g. P) at pH 8·8: ▣—▣, with milk phosphatase (approx. 10 $\mu$g.); □—□, with intestinal phosphatase (approx. 10 $\mu$g.); ■—■, with intestinal phosphatase (approx. 20 $\mu$g.).

AMP (195 $\mu$g. P) at pH 9·3: △—△, with milk phosphatase (approx. 10 $\mu$g.); +—+, with intestinal phosphatase (approx. 5 $\mu$g.)

PC (328 $\mu$g. P) at pH 9·5; ◒—◒, with intestinal phosphatase (approx. 20 $\mu$g.).

Somewhat similar results were obtained with the ADP preparation, as shown in Fig. 2. In this case, however, the inorganic phosphate liberated enzymically after 90 min. amounted to 15 % of the total organic phosphate. Since similar results were obtained with two enzymes, and with intestinal phosphatase at two concentrations (approx. 10 and 20 $\mu$g. protein per tube), the enzymic reaction had effectively reached completion.

AMP was shown to be present in the ADP preparation, and comparison of the acid-labile phosphorus (81 $\mu$g.) with the total organic phosphorus (185 $\mu$g.) indicated that between 10 and 20 % of the organic phosphate was probably present as AMP. The results therefore show that ADP, like ATP, is not hydrolysed by either milk or intestinal phosphatase. The inorganic phosphate liberated is attributed to hydrolysis of contaminant AMP which, as shown in Fig. 2, is rapidly and completely hydrolysed by both phosphatases.

When a preparation of DPN, containing 214 $\mu$g. organic phosphorus per reaction, was examined with both phosphatases under the same experimental conditions as those used with ATP, the results were essentially similar to those obtained with ATP. The inorganic phosphorus (38 $\mu$g.) liberated after 90 min. incubation with 10 $\mu$g. intestinal phosphatase was undoubtedly due to the presence of contaminant AMP. Further evidence that DPN is not hydrolysed by either milk or intestinal phosphatase is presented in another paper (Morton, 1955*b*).

*The effect of certain esterase inhibitors on milk and intestinal alkaline phosphatases.* The experiments were carried out as described under Methods, using approx. 0·55 $\mu$g. of intestinal phosphatase and 2·5 $\mu$g. of milk phosphatase per tube. The results are shown in Table 3. None of the organophosphorus compounds examined inhibited appreciably except at relatively high concentrations, i.e. at greater than $10^{-3}$ M concentration. However, cysteine and iodine caused 72 and 84 % inhibition respectively at $10^{-3}$ M concentration.

## DISCUSSION

*The substrate specificity of alkaline phosphatases*

The results reported in this paper were obtained with enzyme preparations which appear to be substantially homogeneous (Morton, 1952, 1953*a*, 1954). It is clear from Table 1 that both purified enzymes hydrolysed all the true orthophosphate monoesters examined. Related compounds containing a primary orthophosphate group, such as

Table 3. *The effect of certain organophosphorus and other compounds on the hydrolysis of phenyl phosphate by purified alkaline phosphatases*

Most of the organophosphorus compounds were made up as concentrated solutions in ethanol. Dilutions were prepared using veronal–sodium acetate–HCl buffer (0·02 M) at pH 7·8. Then 0·1 ml. of the compound at the appropriate dilution was added to 0·4 ml. of the diluted enzyme in the same buffer at pH 7·8, the reactants mixed and held for 20 min. at room temp. (approx. 18°). The activity test was carried out by addition of 0·5 ml. solution containing phenyl phosphate, magnesium acetate, and ethanolamine–HCl buffer (pH 9·95) and sufficient 0·1 N NaOH so that the final pH of the reaction was 9·95. Final concentrations for the activity test were: disodium phenyl phosphate, 0·02 M; magnesium acetate, 0·01 M; ethanolamine–HCl buffer (0·04 M). Hydrolysis was estimated by determination of phenol or of inorganic phosphate (where cysteine or iodine was present) liberated enzymically in 5 min. at 38°. Each tube contained sufficient enzyme to liberate approx. 2 μmoles phenol (or inorganic phosphate)/5 min. in the absence of inhibitor.

| | Compound | −Log* concn. (M) | Percentage inhibition: Intestinal phosphatase | Percentage inhibition: Milk phosphatase |
|---|---|---|---|---|
| Phosphates | Disodium phosphate | 2 | 25 | — |
| | Trimethyl phosphate | 2 | 0 | 0 |
| | Triphenyl phosphate | 2 | 11 | 15 |
| Polyphosphates | Sodium pyrophosphate | 2 | 36 | — |
| | Tetraethyl pyrophosphate | 2 | 0 | — |
| | | 3 | 0 | 0 |
| | Hexaethyl tetraphosphate | 2 | 36 | — |
| | | 4 | 2 | 0 |
| Phosphites | Sodium hypophosphite | 2 | 0 | — |
| | Sodium phosphite | 2 | 2 | — |
| | Di*iso*propyl phosphite | 2 | 51 | — |
| | | 3 | 10 | 4 |
| | | 4 | 3 | — |
| | | 5 | 0 | — |
| | Tri*iso*propyl phosphite | 2 | 17 | — |
| | | 3 | 4 | — |
| | | 4 | 0 | — |
| | Tri-*n*-butyl phosphite | 3 | 1 | — |
| | Triphenyl phosphite | 3 | 27 | 32 |
| | | 4 | 0 | — |
| Phosphonates | Diethyl phosphonate | 2 | 2 | 0 |
| | Ammonium fluorophosphonate | 2 | 28 | 28 |
| | Di*iso*propyl fluorophosphonate | 3 | 20 | 26 |
| Other compounds | Sodium borate | 2 | 13 | — |
| | Tribenzyl borate | 2 | 38 | 24 |
| | Sodium arsenate | 2 | 74 | — |
| | | 3 | 0 | — |
| | Sodium arsenite | 3 | 13 | — |
| | *p*-Nitrophenyl sulphate | 3 | 0 | — |
| | Cysteine | 2 | 84 | — |
| | | 3 | 72 | — |
| | | 4 | 0† | — |
| | Iodine (in potassium iodide) | 2 | 100 | — |
| | | 3 | 84 | — |
| | | 4 | 0‡ | — |

* Concentration during treatment of enzyme, before activity test.
† 15% activation.
‡ 20% activation.

PEP and PC, were also hydrolysed. However, Table 1 also shows that, under the experimental conditions used, both enzymes failed to hydrolyse any orthophosphate diesters or triesters or inorganic or organic polyphosphates. Hence both enzymes have well-defined substrate specificities and are true orthophosphate monoesterases.

There is no reason for doubting that both phosphatases will hydrolyse all true orthophosphate monoesters. However, the rates of hydrolysis (Fig. 1) and the $K_m$ values (Table 2) vary with different substrates, as observed earlier by Delory & King (1943). As shown by comparison of the values obtained with adenosine 3′- and 5′-phosphates (Table 2), the $K_m$ values (and the rates of hydrolysis) may differ considerably even for closely related compounds.

The classification of alkaline phosphatases as monoesterases by Folley & Kay (1936) was based on observations of more rapid hydrolysis of monothan of di-esters or polyphosphates by crude or partially purified preparations from various tissues. Where the substrate specificity of highly active preparations was adequately examined, however, it was found that YNA, ATP and inorganic pyrophosphate were hydrolysed as well as phosphate monoesters (Schmidt & Thannhauser, 1943). Indeed, such is the pyrophosphatase activity of certain preparations of intestinal alkaline phosphatase that these have been used to obtain evidence for a pyrophosphate link in coenzyme A (Novelli, 1953). However, the results shown in Table 1 indicate that the purified intestinal phosphatase, as well as the milk phosphatase, is devoid of pyrophosphatase activity. Likewise, the extensive hydrolysis of nucleic acids by intestinal phosphatase preparations (Schmidt & Thannhauser, 1943; Zittle, 1946) can now be attributed to contaminant enzymes in the phosphatase preparations. Diesterase activity may similarly accompany the alkaline phosphatase of milk, even after considerable purification, as shown by Zittle & Della Monica (1952).

Since this work was completed and reported elsewhere (Morton, 1952), Schramm & Armbruster (1954) have also shown that a highly active alkaline phosphatase preparation obtained from calf intestinal mucosa hydrolyses diphenyl phosphate and YNA at a rate which is negligible compared with that of the hydrolysis of monophenyl phosphate, glycerophosphate and adenylic acid.

Folley & Kay (1936) classified the enzymes hydrolysing phosphoamides, as distinct from the orthophosphate monoesterases. Gomori (1948) and Holter & Li (1950, 1951) have both claimed to have demonstrated specific tissue phosphoamidases, using phospho-*p*-chloroanilide as substrate. However, as shown in Table 1 and Fig. 2, the naturally occurring phosphoamide PC is rapidly hydrolysed by both the purified milk and intestinal enzymes. Winnick (1947) had earlier suggested that PC is hydrolysed by alkaline phosphatases since the activity of different tissue extracts with PC was found to be proportional to their activity with phenyl phosphate. Since a highly purified acid phosphatase preparation from prostate gland also rapidly hydrolysed PC at pH 5·5 (Morton, 1952), it seems not unlikely that PC is attacked at acid pH by the enzyme of wide substrate specificity rather than by a specific phosphoamidase. Furthermore, PC, like phenyl phosphate, acts as a donor for the transfer reactions catalysed by both milk and intestinal alkaline phosphatases and by prostate-gland acid phosphatase (Morton, 1952, 1953*b*). None of the substrate-specific phosphatases investigated catalysed such a transfer reaction (Morton, 1952), and hence it is unlikely that the observed reactions with PC could be due to specific phosphoamidases.

These findings do not preclude the possibility that true phosphoamidases may exist in certain tissues, but they clearly show that the present classification of phosphoamidases as distinct from orthophosphate monoesterases (Roche, 1950), especially as this relates to activity at alkaline pH values, is not justified by experimental findings. True substrate-specific phosphoamidases have yet to be isolated and described.

Since phosphoamides are hydrolysed by both alkaline phosphatases, it is likely that thiophosphates, which have recently been synthesized (Walsh, 1952), are also hydrolysed by these enzymes. It is to be expected that the –S–P bond in thiophosphates is even more analogous to the –O–P bond in orthophosphates than is the –N–P bond in phosphoamides. Although no true acyl phosphate has been tested, the somewhat related PEP is rapidly hydrolysed by both enzymes, and there is no reason for doubting that the true acyl phosphates would also be hydrolysed. Lipmann (1946) has suggested that acyl phosphates are hydrolysed at alkaline pH by a non-specific phosphatase, as well as by a more specific enzyme.

It should be noted that most of the compounds which were not hydrolysed by the alkaline phosphatases were examined over a wide range of pH conditions. Large amounts of enzymes were used together with extended incubation periods. This seemed particularly necessary in order to establish a negative finding, in view of the dependence of the rate of hydrolysis by alkaline phosphatase on both the pH and the substrate concentration (Morton, 1952).

The results shown in Table 1 and Fig. 2 establish that the milk and intestinal phosphatases have

similar substrate specificities. However, these enzymes are not identical, as has been shown in another paper (Morton, 1955*a*).

*The action of some esterase inhibitors on alkaline phosphatases*

The known inhibitors of alkaline phosphatases (see Roche & Thoai, 1950; Roche, 1950) are relatively unspecific. They fall into the following classes: (*a*) metal-binding agents such as $\alpha\alpha'$-dipyridyl and ethylenediaminetetraacetic acid; (*b*) inhibitory metal salts such as zinc and beryllium chlorides; (*c*) amino-binding agents such as keten, phenyl *iso*cyanate, nitrous acid and formaldehyde, and (*d*) competitive inhibitors of hydrolysis, such as inorganic phosphate. Sizer (1942) also showed that oxidizing agents such as potassium permanganate and iodine inhibit alkaline phosphatase activity. This inhibition was partly reversible.

It was hoped that it would be possible to find specific reagents which would non-competitively inhibit alkaline phosphatases. Since the study of the substrate specificity as already discussed clearly indicated that both the milk and intestinal enzymes are true orthophosphate monoesterases, the investigation of certain esterase inhibitors for this purpose was suggested by Dr M. Dixon.

However, as shown by the results in Table 3, none of the organic phosphates, polyphosphates, phosphonates, phosphites or fluorophosphonates investigated had any marked inhibitory action on either the intestinal or milk enzyme. It is noteworthy, however, that all of the organic phosphites and phosphonates investigated were secondary or tertiary compounds. It is possible that certain primary phosphites or phosphonates may inhibit more strongly.

Many of the organophosphorus compounds listed in Table 3 are extremely toxic, being powerful inhibitors of carboxylic esterases, especially of cholinesterases (McCombie & Saunders, 1946; Webb, 1948; Mackworth & Webb, 1948). Hence the phosphoric esterases, which are only slightly inhibited by these compounds, must differ considerably from the carboxylic esterases. The organophosphorus compounds which are inhibitory to carboxylic esterases are apparently hydrolysed by these enzymes, giving rise to inactive phosphorylated enzyme derivatives (see Davies, 1954, for review). However, the phosphoric esterases do not appear to hydrolyse, or combine with, organophosphorus compounds other than the primary esters of orthophosphoric acid, and hence are not inhibited by the organophosphorus compounds listed in Table 3. It should be noted that Webb (1948) had previously shown that crude kidney alkaline phosphatase was only slightly inhibited by relatively high concentrations of DFP.

The most effective inhibitors among the compounds investigated were iodine and cysteine, both of which caused strong inhibition of the milk and intestinal enzymes at $10^{-3}$M inhibitor. The inhibition by cysteine is complicated by the possibility of metal chelation and by the fact that, since the enzyme and cysteine were held at pH 7·8 for 20 min. before addition of the substrate, much of the cysteine may have been oxidized to cystine.

The inhibitory action of iodine is particularly interesting. It is very probably due to the iodination of phenolic hydroxyl groups of the enzyme, as suggested by Sizer (1942). It seems likely that certain tyrosine groups of intestinal phosphatase may be directly involved in the formation of an enzyme–substrate complex. Some evidence in support of this hypothesis has been obtained by using the method of Dixon (1953*b*) to examine the $pK_m$ versus pH relationship for hydrolysis of phenyl phosphate by intestinal phosphatase (Morton, 1952; Dixon, 1953*b*). This work will be presented in detail in a subsequent paper.

Sodium arsenate, at $10^{-2}$M, caused a much greater inhibition of the intestinal phosphatase than did the same concentration of sodium phosphate. Since sodium phosphate is a competitive inhibitor of hydrolysis of organic phosphates (Jacobsen, 1932, 1933), it is probable that sodium arsenate is also a competitive inhibitor. In this regard, it should be noted that the activity test was carried out with very high substrate concentration and at highly alkaline pH in order to reduce competitive inhibition to a minimum. Under other experimental conditions, inhibition by arsenate may be much greater. It is probable that the phosphatases would have a greater affinity for organic derivatives of arsenic acid than for inorganic arsenate. Thus, organic derivatives of arsenic acid would seem worthy of investigation as useful competitive inhibitors of alkaline (and acid) phosphatases.

## SUMMARY

1. The substrate specificity of alkaline phosphatases, purified from cow's milk and calf intestinal mucosa, has been investigated, using a wide range of compounds under several different experimental conditions.

2. All true orthophosphate monoesters were rapidly hydrolysed by both enzymes, as well as the orthophosphoamide, phosphocreatine, and the enolic phosphate, phosphoenolpyruvate.

3. Sodium pyrophosphate, sodium hexametaphosphate, diphenyl pyrophosphate, ATP, ADP and DPN were not hydrolysed by either enzyme, showing absence of any pyrophosphatase activity. Absence of diesterase activity was shown from studies with partially degraded yeast nucleic acid,

both in the presence and absence of added ribonuclease.

4. A number of organic polyphosphates, phosphates, phosphites, phosphonates and fluorophosphonates were found to cause only slight inhibition of milk or intestinal phosphatase at $10^{-2}$M concentration under the experimental conditions used. However, iodine and cysteine at $10^{-3}$M concentration both caused strong inhibition of the two phosphatases. A possible mechanism for the inhibition by iodine is discussed.

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# The Action of Purified Alkaline Phosphatases on Di- and Tri-Phosphopyridine Nucleotides

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The conversion of triphosphopyridine nucleotide (TPN) into diphosphopyridine nucleotide (DPN) was first reported by von Euler & Adler (1938). The nature of this reaction was not apparent until Kornberg & Pricer (1950*a*) showed that the third phosphate group of TPN was esterified at either the 2′- or 3′-position of the ribose moiety attached to adenine. Hydrolytic cleavage of this esterified phosphate group could thus account for the conversion of TPN into DPN.

It seemed likely that such a reaction would be catalysed by alkaline phosphatases which had been purified from cow's milk and calf intestinal mucosa (Morton, 1950, 1953*a*, 1954). It had been established that these enzymes acted on monoesters and amides of orthophosphoric acid, but not on diesters

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or on pyrophosphates (Morton, 1955*a*). Hydrolysis by these enzymes would thus confirm the presence of a phosphate group in TPN esterified to ribose as proposed by Kornberg & Pricer (1950*a*). Hence the action of the purified enzymes on TPN was investigated, as well as the possible enzymic and non-enzymic synthesis of TPN from DPN by transphosphorylation from phosphocreatine and phenyl phosphate. Most of this work was carried out in 1950 and, although not previously published, has been described in detail elsewhere (Morton, 1952*a*).

## MATERIALS AND METHODS

### *Enzymes*

*Alkaline phosphatases.* These were purified from cow's milk (Morton, 1950, 1953*a*) and from calf intestinal mucosa (Morton, 1950, 1954). The method of storage and handling of these preparations is described in another paper (Morton, 1955*b*).

*Alcohol dehydrogenase.* This was crystallized from baker's yeast as described by Racker (1950). A crystalline enzyme from liver, kindly supplied by Dr E. C. Slater, was used in some of the initial experiments.

*Lactic dehydrogenase of muscle.* This was prepared from rabbit skeletal muscle essentially as described by Askonas (1951).

iso*Citric dehydrogenase.* This was prepared from pig heart essentially as described by Grafflin & Ochoa (1950).

*Glucose 6-phosphate (G 6-P) dehydrogenase.* This was a commercial preparation (Sigma Chemical Co.).

### *Buffers*

*Monoethanolamine.* This was purified and buffers were prepared as described by Morton (1955*b*).

*Aminotrishydroxymethylmethane* (Tris). A commercial preparation (Sigma Chemical Co.) was used without further purification, and buffers were prepared as recommended by the manufacturers.

### *Substrates*

*Phosphocreatine.* This was prepared according to Ennor & Stocken (1948).

*Diphosphopyridine nucleotide.* A number of different preparations have been used, with essentially similar results. Initially, preparations assaying 33% DPN, prepared by the late Mr J. Morgan, and 50% DPN, kindly supplied by Dr E. C. Slater, were used, and in later work a commercial preparation of 90% purity (Sigma '90', Sigma Chemical Co.).

*Triphosphopyridine nucleotide.* Two preparations, one assaying 19% TPN (kindly supplied by Dr E. C. Slater) and one of 48% TPN (Sigma Chemical Co.), have been used with similar results. Both preparations were free of DPN.

### *Methods*

All measurements were made at room temperature (15–19°), using a Beckman spectrophotometer, Model DU, with 1 cm. quartz cuvettes. A value of $6{\cdot}22 \times 10^3$ at 340 m$\mu$. has been used for the molar extinction coefficient of reduced DPN and reduced TPN (Horecker & Kornberg, 1948).

*Estimation of DPN.* This was measured by reduction with alcohol dehydrogenase essentially as described by Racker (1950). The test cuvette contained ethanolamine–HCl buffer (pH 9·3 at 20°) 0·142 mmole; aldehyde-free ethanol, 2 mmoles; and sufficient alcohol dehydrogenase to cause complete reduction of 0·02 $\mu$mole DPN within 3 min. The final volume was usually 3 ml.

*Estimation of reduced DPN.* This was measured by oxidation with muscle lactic dehydrogenase. The cuvette contained, in 3 ml., 150 $\mu$moles tris buffer, pH 8·6; 20 $\mu$moles pyruvic acid; and sufficient enzyme to oxidize 0·02 $\mu$mole of reduced DPN in about 5 min. At this dilution of enzyme there was no reaction with 0·1 $\mu$mole reduced TPN in 15 min. Hence it was possible to measure reduced DPN in the presence of reduced TPN, as previously described by Kornberg (1950).

*Estimation of TPN.* This was generally measured by reduction with G 6-P dehydrogenase. The test cuvette contained, in 3 ml., 150 $\mu$moles tris buffer, pH 8·6 at 20°; 30 $\mu$moles G 6-P; and sufficient G 6-P dehydrogenase to cause complete reduction of 0·05 $\mu$mole of TPN in about 5 min. In some cases TPN was reduced with *iso*citric dehydrogenase. The test cuvette contained, in 3 ml., glycylglycine–HCl buffer (pH 7·4; 100 $\mu$moles); sodium DL-*iso*citrate (approx. 10 $\mu$moles); manganous chloride (1·8 $\mu$moles); and sufficient enzyme to cause complete reduction of 0·05 $\mu$mole of TPN in about 10 min.

In consequence of observations to be described, TPN has routinely been estimated as follows. The test cuvette contains ethanolamine–HCl buffer and ethanol as described above for estimation of DPN, and, in addition, 30 $\mu$moles magnesium chloride. Yeast alcohol dehydrogenase is added, and any change in optical density, due to DPN, is measured. Then 0·1 ml. of a solution containing about 10 $\mu$g. purified intestinal alkaline phosphatase (Morton, 1954) is added, and the further change in optical density at 340 m$\mu$., due to the quantitative conversion of TPN into DPN, is measured. This gives an accurate estimation of TPN. The method has the advantage that both DPN and TPN may be determined in the one cuvette, and use is made of two enzymes which may be readily obtained in a highly purified and stable condition.

## RESULTS

### *Conversion of oxidized TPN into DPN*

This experiment was carried out by following the enzymic reduction of DPN at 340 m$\mu$., using alcohol dehydrogenase as described under Methods. As shown in Fig. 1, 0·16 $\mu$mole of DPN added to the test cuvette was rapidly and completely reduced. When 0·17 $\mu$mole TPN was added to a similar cuvette, however, there was no change in optical density (Fig. 1, curve *B*), up to 5 min. At this time, 0·17 ml. of a solution containing approx. 2·6 $\mu$g. purified intestinal alkaline phosphatase was added. As shown in Fig. 1, curve *B*, there was an immediate increase in optical density. After 30 min. at room temperature (approx. 15°), the curve approached the theoretical value to be expected for quantitative conversion of the TPN into DPN.

Comparison of the curves *A* and *B* (Fig. 1) show that the alkaline phosphatase was limiting in the experiment represented by curve *B*.

The quantitative conversion of TPN into DPN shows that the purified phosphatase has no action on DPN. This was further confirmed by incubating 0·15 $\mu$mole DPN with 2·6 $\mu$g. intestinal phosphatase at pH 9·0 in ethanolamine–HCl buffer (0·04 M) and magnesium acetate (0·01 M) before addition to the test cuvette. The DPN was subsequently fully reduced by the alcohol dehydrogenase, confirming that the phosphatase has no action on DPN (Morton, 1955*a*).

The quantitative conversion of TPN into DPN has also been obtained using purified milk alkaline phosphatase instead of intestinal phosphatase. Since the use of alkaline phosphatase and alcohol dehydrogenase thus leads to the complete reduction of TPN, the method has been routinely used for the estimation of TPN (see under Methods). The substantial agreement with enzymic estimation of TPN with both *iso*citric dehydrogenase and G 6-P dehydrogenase is shown in Table 1.

Fig. 1. The conversion of oxidized TPN into oxidized DPN by intestinal alkaline phosphatase. The reaction mixture contained ethanolamine–HCl buffer, pH 9·3 (0·142 mmole), ethanol (2 mmole), alcohol dehydrogenase and, for curve *B*, magnesium chloride (30 $\mu$moles) in 3 ml. Curve *A* shows the change in optical density after addition of 0·16 $\mu$mole DPN; curve *B*, after addition of 0·17 $\mu$mole TPN. At the arrow (curve *B*), purified intestinal phosphatase (approx. 2·6 $\mu$g.) was added. The optical density for complete conversion of TPN into DPN is indicated by the line *Y*.

Table 1. *Comparative estimates of TPN by different enzymic methods*

TPN, as estimated originally from assay using *iso*citric dehydrogenase, was added to each cuvette, and the contents assayed by different procedures as described under Methods. The final volume in each cuvette was 3 ml.

| TPN ($\mu$mole) | Change in optical density at 340 m$\mu$., using: Alkaline phosphatase with alcohol dehydrogenase | G 6-P dehydrogenase | *iso*Citric dehydrogenase |
|---|---|---|---|
| 0·108 | 0·232* | 0·228 | 0·233 |
| 0·197 | 0·405† | 0·401 | 0·407 |
| 0·218 | 0·452† | 0·451 | 0·450 |

* With milk alkaline phosphatase.
† With intestinal alkaline phosphatase.

*Conversion of reduced TPN into reduced DPN*

The estimation of reduced DPN was carried out spectrophotometrically as described under Methods. On addition of reduced DPN (0·22 $\mu$mole) to the test cuvette containing muscle lactic dehydrogenase, it was rapidly and completely oxidized, as shown in Fig. 2, curve *A*. When reduced TPN was added to a similar cuvette, there was no change in optical density (curve *B*), up to 5 min. The addition of 0·1 ml. solution containing approx. 2·5 $\mu$g. intestinal phosphatase caused rapid and complete conversion of the reduced TPN into DPN, as shown by curve *B* of Fig. 2. In this reaction, the muscle

Fig. 2. The conversion of reduced TPN into reduced DPN by intestinal alkaline phosphatase. The reaction mixture contained tris buffer, pH 8·6 (150 $\mu$moles), pyruvic acid (20 $\mu$moles) and diluted muscle lactic dehydrogenase. Curve *A* shows the change in optical density after addition of 0·22 $\mu$mole of reduced DPN; curve *B*, after addition of 0·22 $\mu$mole of reduced TPN. At the arrow (curve *B*), purified intestinal phosphatase (approx. 2·5 $\mu$g.) was added.

lactic dehydrogenase was apparently limiting the rate. This resulted from dilution of the lactic dehydrogenase to the point at which there was no measurable reaction with reduced TPN.

*Attempted transphosphorylation with oxidized DPN as acceptor*

Both purified intestinal and milk phosphatases catalyse a direct transfer of the phosphate group from certain donor compounds to suitable acceptors (Morton, 1952*b*, 1953*b*). Synthesis of TPN by transphosphorylation to DPN was therefore investigated.

It has previously been shown that the transferase reaction catalysed by purified alkaline phosphatase is due to competition of the acceptor molecule with water (Morton, 1952*a*, 1953*b*). In attempting to obtain appreciable synthesis of TPN, therefore, it was necessary to use high concentrations of DPN.

In the initial experiment, the reaction mixture (0·25 ml.) contained phenyl phosphate (0·025 M) as donor; magnesium chloride (0·01 M); DPN (0·07 M); ethanolamine–HCl buffer (0·04 M) at pH 9·6; and intestinal phosphatase (about 1·5 $\mu$g.). The control contained all components except phosphatase. After incubating for 30 min. at 38°, the tubes were heated to 100° for 30 sec., and TPN was estimated, using G 6-P dehydrogenase as described under Methods. There was no appreciable synthesis of TPN.

This reaction was repeated essentially as described above, but with the addition of G 6-P (0·04 M) and G 6-P dehydrogenase to each tube, and with tris buffer (0·05 M), at pH 8·8, in place of ethanolamine buffer. Under these conditions, any TPN synthesized should be immediately reduced. Moreover, even if TPN was formed and subsequently re-converted into DPN by hydrolysis, the reduced DPN would provide evidence of synthesis of TPN. However, there was no measurable formation of TPN after 30 min. incubation at 38°.

It has previously been shown that a non-enzymic transphosphorylation of glucose from phosphocreatine occurs at pH 4·0 (Morton, 1952*a*, *b*). It seemed desirable to investigate whether DPN could act as a phosphate acceptor to form TPN under similar conditions. For this purpose, 7·6 mg. of DPN (90 % pure) and 12·3 mg. of phosphocreatine were weighed into a small tube and 0·2 ml. of 0·5 M sodium acetate buffer, pH 4·2, was added. After the components had dissolved, 0·1 ml. was removed immediately and held in crushed ice. The remaining solution was incubated for 30 min. at 38°, after which 0·1 ml. was removed. TPN was then estimated in both samples, using G 6-P dehydrogenase. There was no measurable synthesis of TPN.

## DISCUSSION

It has previously been shown that the alkaline phosphatases purified from cow's milk and calf intestinal mucosa are true orthophosphomonoesterases, without action on phospho di-esters or pyro- or poly-phosphates (Morton, 1955*a*). The action of these purified enzymes, of defined specificity, on TPN as described here therefore leaves no doubt that TPN contains an esterified phosphate group which is readily hydrolysed, giving rise to DPN and inorganic phosphate.

The quantitative conversion of TPN into DPN with purified intestinal phosphatase (Fig. 1) was first observed in 1950, in collaboration with Dr E. C. Slater, shortly after the elegant formulation of the structure of TPN by Kornberg & Pricer (1950*a*). This study provided confirmation of the finding by Kornberg & Pricer that the third phosphate group of TPN was attached by an ester, rather than by an anhydride, link.

Kornberg & Pricer (1950*b*) observed that crude potato extracts, like yeast maceration juice (von Euler & Adler, 1938), would catalyse the conversion of TPN into DPN. That this reaction was due to a hydrolytic cleavage required investigation with purified enzymes of defined specificity as herein described. After this work had been completed, reports appeared of the conversion of TPN into DPN by partially purified prostate gland acid phosphatase (Kaplan, Colowick & Ciotti, 1952) and by kidney extracts (Sanadi, 1952). Sanadi (1952) partially purified the enzyme, which he designated as TPNase, although he pointed out that the properties of this enzyme closely resembled those of alkaline phosphatase. Consideration of the properties of Sanadi's enzyme with those of alkaline phosphatases (Morton, 1954, 1955*b*) leaves no reasons for doubting the identity with kidney alkaline phosphatase.

The failure to obtain synthesis of TPN from DPN by transphosphorylation was not unexpected. As shown previously (Morton, 1953*b*) both phosphatases exhibit a specificity toward the acceptor, and only some, but not all, compounds containing hydroxyl groups can compete with water for the transferred group from the donor compound. Both the structure and the size of the acceptor molecule seem important, since glucose and fructose, but not sucrose, act as acceptors for milk and intestinal phosphatases (Morton, 1952*a*, 1953*b*). DPN may well fail to act as an acceptor for similar reasons although it should be pointed out that the maximum concentration of DPN achieved in the present studies was well below that required to eliminate entirely the possibility of DPN acting as a phosphate acceptor to form TPN. It is noteworthy that Sanadi (1952) failed to obtain any

appreciable synthesis of TPN from DPN and inorganic phosphate, using the kidney enzyme discussed above. These negative findings with the phosphatases underline the importance of the specific phosphokinase described by Kornberg (1950) which catalyses the synthesis of TPN from DPN, using adenosine triphosphate as the specific donor compound.

## SUMMARY

1. Purified alkaline phosphatases from cow's milk and calf intestinal mucosa quantitatively convert TPN into DPN. DPN is not hydrolysed by either enzyme.

2. These results confirm that the third phosphate grouping of TPN is esterified (to ribose), since the action of both purified phosphatases is restricted to phospho mono-esters and amides.

3. Under the experimental conditions used, there was no measurable synthesis of TPN from DPN, either by enzymic transfer from phenyl phosphate by intestinal alkaline phosphatase or by non-enzymic transfer from phosphocreatine at pH 4·2.

4. By using alcohol dehydrogenase, followed by alkaline phosphatase, DPN and TPN can be estimated spectrophotometrically in the same solution.

I wish to thank Dr M. Dixon, F.R.S., for his encouraging interest and advice during the course of this work, which was largely carried out while holding a Travelling Scholarship awarded by the Gowrie Scholarship Trust of Australia. I am grateful to Dr E. C. Slater for his assistance with some of the experiments.

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# Phosphorylations in Rabbit Liver *in vivo*

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A study has been made of the time-course of the distribution of tracer phosphate among the acid-soluble P compounds of the liver, primarily to determine the extent to which correlations may be obtained *in vivo* with the known enzymic reactions involving these phosphorylated intermediates, and also to study some of the dynamic aspects of these processes. Such an investigation has been made previously (Sacks, 1951) in which the separations of the various P compounds present in a trichloroacetic acid (TCA) extract of liver were made on the basis of the solubilities of the barium salts and the relative ease of hydrolysis. Some of the desired separations were not possible by the methods then available; for example a separation of adenosine diphosphate (ADP) and adenosine triphosphate (ATP) could not be made. As a consequence of the inadequacy of the methods, some of the findings were difficult to correlate with the known enzymic reactions. In particular, the results obtained failed to show that either glucose 1-phosphate (G 1-P) or ATP met the requirements of the postulates of Zilversmit, Entenman & Fishler (1943) for being the precursor of the P of glucose 6-phosphate (G 6-P).

The introduction of ion-exchange chromatography by Cohn & Carter (1950) for the separation of adenosine monophosphate (AMP), ADP and ATP, and the more recent development by Khym & Cohn (1953) of ion-exchange procedures for the separation of the phosphorylated sugars as the borate complexes, have made possible many of the

isolations necessary for such a study. These methods have been applied to experiments on rabbits, in order to obtain data for comparison with those of the previous experiments on rats. The results obtained have resolved some of the anomalies of the earlier data, so that a better correlation is possible between the findings *in vivo* and those of the known enzymic mechanisms. In addition, inorganic pyrophosphate (PP) is found to be present in significant amount in liver, and some functions of this compound have been suggested. Certain of the dynamic aspects of the metabolism of the phosphorylated intermediates have also been brought to light. Results on the time-course of the specific radioactivity have been obtained for orthophosphate, PP, AMP, phosphoglyceric acid (PGA), α-glycerophosphate (α-GPA), G 1-P, G 6-P, 4-carboxy-4-hydroxy-2-phosphoadipic acid (PHCA), and for each of the P groups of ADP and ATP. Some specific activity results have also been obtained for fructose 1-phosphate (F 1-P) and uridine 5′-phosphate (UMP). In each case except that of PHCA the identity of the compound on which radioactivity measurements were made, isolated by the ion-exchange procedure, has been established by parallel experiments in which the known pure compound has been adsorbed on and eluted from the ion-exchange resin, and by other appropriate tests.

## EXPERIMENTAL

The experiments were carried out on young male New Zealand white rabbits weighing 1·7–2·4 kg. Free access to food and water was allowed up to the time of injection of the tracer phosphate, and access to water was allowed after the injection in the experiments of 4 and 6 hr. duration. The tracer phosphate was injected intravenously in dose of 50–100 μc/kg. body weight, in 0·5–2·0 ml. of normal saline/kg. to which had been added 5 μg./ml. carrier P as $Na_2HPO_4$. Anaesthesia was induced just before the time of death by pentobarbital injected intravenously, supplemented by ether inhalation. At the appropriate time, a blood sample was drawn by cardiac puncture into a syringe containing heparin powder, and immediately thereafter the liver was excised and dropped into a large volume of a mixture of solid $CO_2$ and ether. The blood sample was centrifuged at 1° immediately after it was drawn, to minimize transfer of tracer P from plasma to corpuscles (Gourley & Gemmill, 1950), and the proteins of the separated plasma were precipitated by the addition of 9 vol. 5% TCA. Small volumes of the filtrate were taken for determination of P, and a large volume for the preparation of a sample for counting.

Further operations on the frozen liver were carried out in a cold room maintained at 1–2°. The extraction of the acid-soluble P compounds, removal of glycogen, and separation into 'alkaline-earth soluble and insoluble fractions' were carried out as previously described (Sacks, 1949). The various P compounds present in the 'alkaline-earth-insoluble' fraction were separated by ion-exchange chromatography by a modification of the procedure described previously (Sacks, Lutwak & Hurley, 1954), made necessary because two of the nucleotide-containing eluates contained P compounds in addition to those responsible for the absorption in the ultraviolet shown by these solutions. The separations of the compounds present in the alkaline earth-soluble fraction were made initially by ion-exchange chromatography of the borate complexes, by a modification of the method of Khym & Cohn (1953).

Each P compound as isolated was converted into orthophosphate by the appropriate hydrolytic or wet-ashing procedure, the orthophosphate was precipitated as $MgNH_4PO_4$ and the precipitate separated by filtration through a sintered-glass filter funnel. The precipitate was then dissolved in a small volume of 5% TCA, the solution made up to suitable volume and samples were taken for determination of orthophosphate by the method of Fiske & Subbarow (1925). A suitable sample, containing 250–350 μg. orthophosphate, was taken for conversion into phosphomolybdate for counting. Where necessary, carrier P was added to the solution before precipitation, to bring the weight of P in the counting sample within this range. The counting was done with a methane flow counter operating in the proportional range. The details of these procedures have been given previously (Sacks, 1951).

### *Separation of alkaline-earth insoluble compounds*

All operations on this fraction in which acid solutions were involved were carried out in the cold room, except for the measurements in the spectrophotometer. The material was dissolved in the minimum volume of 0·2 N-HCl, and the solution freed from calcium and barium by passage through a column of Dowex-50 cation-exchange resin in the sodium form. The resulting solution was brought to pH 8–9 by the addition of dilute ammonia, and made up to a volume sufficient to reduce the chloride concentration below 0·01 M, the limiting value for complete adsorption of the anions on Dowex-1 resin (Cohn & Carter, 1950). Small samples were taken for determination of orthophosphate and of total P, and for adenine equivalent, by measurement of optical densities at 260 and 280 mμ. in a Beckman Model DU spectrophotometer. The solution was then passed over a column of Dowex-1 anion-exchange resin, 17–18 cm. high by 1·1 cm. in diameter, to adsorb the P compounds present. The resin used was the ordinary 10% crosslinked material, 200–400 mesh. The column was then washed with water, and the elution begun, at the rate of approximately 50 ml./hr., with 2 hr. collections made by an automatic fraction collector. Orthophosphate was completely eluted with 1 l. of the mixture of 0·01 M-$K_2B_4O_7$ and 0·025 M-$NH_4Cl$, used by Khym & Cohn (1953) in their work on the separation of the sugar phosphates as the borate complexes. The column was then buffered by 500 ml. of 0·01 M-$NH_4Cl$ (Volkin & Carter, 1951), and the elution of the nucleotide fractions begun. The progress of the elution was followed by measurements of the optical density of the eluates at 260 and 280 mμ. Fig. 1 is a representative elution pattern, and gives the sequence of eluents used and the materials obtained with each eluent.

The eluate representing a single peak in the elution pattern was made alkaline with $NH_3$, and the P compound or compounds present adsorbed on a short column of the Dowex-1 resin, 2–3 cm. high by 1·1 cm. in diameter. Those eluates in which the chloride concentration exceeded

0·01 M were diluted two- to three-fold before this readsorption. The P compounds were then eluted from the short columns with small volumes of eluents of higher ionic strength and acidity than those used for the original separation. In this way a high degree of concentration was achieved, so that material originally contained in 1–2 l. was now present in 50–75 ml. of solution. 0·02 N-HCl was used to elute the AMP and UMP from the short columns, and N-HCl for all the other fractions except ATP. For this the eluent was 0·02 N-HCl plus 0·5 M-KCl, to give directly the concentration of potassium ion for the optimum activity of ATPase.

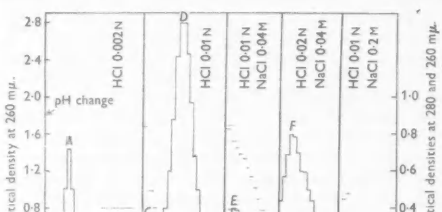


Fig. 1. Elution pattern of nucleotide derivatives of barium-insoluble fraction of acid-soluble P compounds of rabbit liver. Continuous line, optical density of eluate at 260 mμ.; dotted lines, ratio of optical densities at 280 and 260 mμ. Elution peaks represent: *A*, AMP; *B*, UMP; *C*, mixture of compounds; *D*, ADP; *E*, mixture; *F*, ATP; *G*, mixture of polyphosphate derivatives. Peak *C* also contains PGA; peak *E* also contains PP, PHCA, and another compound of unknown nature.

Before the identity of peak *B* of Fig. 1 with UMP was established, this material was saved in the attempt to obtain enough material for characterization. The results on the radioactivity of UMP in Table 1 were obtained from the smaller number of experiments carried out after an authentic sample of UMP became available for comparison.

The concentrated eluates containing the AMP and UMP were wet-ashed with nitric and sulphuric acids for conversion into orthophosphate, which was then precipitated as $MgNH_4PO_4$.

The first 600 ml. of eluate obtained with 0·01 N-HCl, peak *C* of Fig. 1, contained PGA and some of the nucleotide derivatives that Hurlbert, Schmitz, Brumm & Potter (1954) isolated from liver by ion exchange from Dowex-1 resin in the formate form with formic acid–ammonium formate eluents. These nucleotide materials were removed from the short-column eluate with acid-washed Norit charcoal (Fiske, 1934), and the filtrate, which showed no absorption in the ultraviolet, wet-ashed for conversion of the PGA present into orthophosphate.

The short-column eluate which contained the ADP was heated in a boiling-water bath for 20 min. to hydrolyse the one acid-labile P group. This was isolated as $MgNH_4PO_4$, and the acid-stable P group then isolated from the filtrate by wet ashing.

The eluate obtained with 0·01 N-HCl plus 0·04 M-NaCl, peak *E* of Fig. 1, has a yellow colour, much more intense than can be accounted for by the quantities of riboflavin derivatives that may be present. It contains some of the nucleotide derivatives isolated by Hurlbert *et al.* (1954) and, with rabbit liver, three other P compounds, which do not absorb in the ultraviolet. The concentrated eluate from the short column containing this fraction was treated with Norit to remove nucleotides, and the filtrate neutralized to phenolphthalein with powdered barium hydroxide. The precipitate which formed was collected by centrifuging, washed with water, and treated with N acetic acid. The major portion dissolved, and the residue was assumed to be the barium salt of PHCA, on the basis that the dissociation constants of two of the carboxyl groups might be expected to be higher than that of acetic acid. This residue was wet-ashed for conversion into orthophosphate.

It was known from some preliminary experiments on rat liver that this eluate also contained PP. The identification as PP was made by parallel experiments with known PP, which showed the same elution characteristics from the resin and the same rate of hydrolysis by N-$H_2SO_4$ at 100°. A sample of the PP fraction from the liver of a rat which had been injected with tracer P was mixed with known PP, readsorbed and eluted from a Dowex-1 column, and all the $^{32}P$ present was found in a single fraction. This material was then precipitated, first as calcium salt, then as silver salt and finally as barium salt. Samples of each salt were converted into orthophosphate, and the same specific radioactivity was found for all three.

The fraction from rabbit liver was found to contain a considerable amount of a not easily hydrolysable P compound in addition to PP. Consequently the co-precipitation experiments were not carried out, as they would not have given significant results.

Fructose 1:6-diphosphate, if present, would also appear in this eluate (personal communication from Dr W. E. Cohn, confirmed with the known compound). Tests for fructose by the method of Roe (1934) on samples of the short-column eluate of this fraction, equivalent to 3–4 g. of liver tissue, were uniformly negative.

$P^{\gamma}$ of ATP was hydrolysed by an ATPase preparation from rabbit muscle, with 0·01 M-$CaCl_2$ and glycine buffer, pH 9·2. Over 95% hydrolysis of $P^{\gamma}$ was obtained. In the precipitation of this as $MgNH_4PO_4$, the remaining ATP was adsorbed on the precipitate. The amount of this adsorbed P was determined, and allowed for in the weight of the counting sample taken, as the procedure for the preparation of the counting sample resulted in the hydrolysis of the labile P of ATP. No corrections to the radioactivity measurements were necessary as the differences between measured specific radioactivities of $P^{\gamma}$ and $P^{\beta}$ were within the standard deviation of the counts. $P^{\beta}$ of ATP was isolated by hydrolysis, and $P^{\alpha}$ by wet-ashing.

### *Separation of 'alkaline-earth-soluble' compounds*

This fraction was found to contain considerable AMP and UMP and traces of other nucleotide derivatives in addition to the sugar phosphates and α-GPA. When the solution of this fraction in dilute HCl was treated with Dowex-50 resin in the sodium form to remove Ba and Ca, then made slightly alkaline with $NH_3$, an appreciable portion of the

total P was not adsorbed on the Dowex-1 resin. This difficulty disappeared when the Dowex-50 was used in the hydrogen form. Some nucleotide material was adsorbed on the Dowex-50, but this was unimportant, as the nucleotide fraction was discarded.

The G 1-P and α-GPA were eluted together from Dowex-1 columns, 17–18 cm. high by 1·1 cm. in diameter, with the mixture of 0·01 M-$K_2B_4O_7$ and 0·025 M-$NH_4Cl$ used by Khym & Cohn (1953). Their finding that there was considerable overlapping in the elution of these two compounds was confirmed. The entire volume of eluate containing these compounds was concentrated *in vacuo* to about 25 ml., at a bath temperature of 45–50°. The solution was stored overnight in the refrigerator, filtered from crystals of the tetraborate, and magnesia mixture added. Usually an appreciable amount of $MgNH_4PO_4$ was obtained, which was shown not to arise either from impurities in the eluting reagents or from hydrolysis of G 1-P. The latter point was established by the finding that the specific radioactivity of this P was much lower than that of the G 1-P. The P of G 1-P was isolated after hydrolysis at 100° for 15 min. in solution made 0·1 N in $HNO_3$. The P of α-GPA was isolated after conversion into glycolaldehyde phosphate and subsequent hydrolysis, by the periodate method of Leva & Rapoport (1943).

The elution of G 6-P was carried out with the mixture of 0·025 M-$NH_4Cl$, 0·0025 M-$NH_4OH$ and 0·001 M-$K_2B_4O_7$, used by Khym & Cohn (1953) for this purpose. The eluate was concentrated to a small volume, and the orthophosphate isolated after wet-ashing in the usual manner.

Tests with authentic F 1-P showed that the elution pattern of this substance was indistinguishable from that of F 6-P, and Khym & Cohn (1953) found that their elution did not effect a complete separation of F 6-P from R 5-P. For these reasons, no further attempts at differential elution were made after the removal of the G 6-P from the Dowex-1 columns. Instead, all remaining P compounds present were stripped from the column by 50 ml. 0·1 N-HCl, nucleotides were removed by Norit, and samples of the Norit filtrate taken for determination of pentose by the method of Mejbaum (1939) and of fructose by the method of Roe (1934). In many cases, most of the fructose found in the original solution of the alkaline-earth-soluble fraction could not be recovered. The ribose determinations indicated the presence of about 0·05 m-mole of R 5-P/kg. wet weight of liver.

Where enough F 1-P was present for an adequate counting sample, the solution was made N in nitric acid, and heated for 40 min. in a boiling-water bath (Tankó & Robison, 1935). This sufficed for complete hydrolysis of F 1-P. The hydrolysis of R 5-P under these conditions, about 5%, would give at most a 5% error in the specific radioactivity value for the P of F 1-P. In this case it was necessary to precipitate the orthophosphate directly as phosphomolybdate, and make the P determination after counting, because the small amount of P present could not be recovered quantitatively as $MgNH_4PO_4$. To avoid interference of chloride with the precipitation of phosphomolybdate, this was removed by the addition of slightly less than the calculated amount of $AgNO_3$. The P found on the precipitate agreed with the fructose value within 3–5%.

Isolation of the P of R 5-P was not attempted because of the small quantity present and the probability that other P compounds, such as propanediol phosphate and β-GPA, were present.

## RESULTS AND DISCUSSION

The amounts of the various P compound found and the specific radioactivity measurements are given in Table 1. The value for AMP represents only that portion co-precipitated in the alkaline-earth-insoluble fraction. More AMP and UMP were

Table 1. *Concentration of acid-soluble* P *compounds in rabbit liver, and time-course of specific radioactivities of* P

Concentrations are as m-moles/kg. wet weight, mean and range. Radioactivity measurements as counts/min./mg. P, calculated to standard dose of $1 \times 10^6$ counts/min. injected/kg. body weight. Figures are means and standard error of mean.

| Hr. after $^{32}P$ injection | | 0·5 | 1 | 2 | 4 | 6 |
|---|---|---|---|---|---|---|
| No. of animals | | 4 | 5 | 5 | 4 | 4 |
| P fraction | Concn. | Specific radioactivity | | | | |
| Plasma inorganic | 2·05 (1·63–2·87) | 19450±750 | 7700±280 | 4100±125 | 2275±150 | 1350±75 |
| Liver inorganic | 5·36 (3·6–7·35) | 3500±170 | 3650±240 | 2925±200 | 2300±150 | 1750±115 |
| AMP | 0·37 (0·15–1·00) | 1100±70 | 1350±100 | 1575±70 | 1550±70 | 1350±95 |
| Labile P of ADP | 1·30 (0·80–1·80) | 1950±105 | 2450±200 | 2400±120 | 2250±70 | 1750±100 |
| Stable P of ADP | — | 285±14 | 625±30 | 975±90 | 1300±95 | 1500±60 |
| Pγ of ATP | 1·08 (0·35–1·42) | 2275±70 | 2575±240 | 2350±90 | 2425±95 | 1575±50 |
| Pβ of ATP | — | 2275±70 | 2550±120 | 2475±115 | 2275±70 | 1700±50 |
| Pα of ATP | — | 440±30 | 1000±75 | 1300±115 | 1250±100 | 1300±170 |
| Pyrophosphate | 0·41 (0·1–1·1) | 2550±210 | 2825±210 | 2375±310 | 2225±60 | 1800±60 |
| PGA | 0·24 (0·10–0·45) | 775±35 | 1050±90 | 1025±50 | 1050±30 | 920±30 |
| PHCA | 0·42 (0·05–1·20) | 2250±170 | 2650±270 | 2300±215 | 2200±80 | 1450±190 |
| G 1-P | 0·20 (0·10–0·61) | 1075±90 | 2050±120 | 2150±115 | 1850±150 | 1400±150 |
| G 6-P | 0·20 (0·10–0·60) | 1575±100 | 2050±210 | 2250±110 | 1925±130 | 1650±75 |
| F 1-P | — | — | 1550* | 1600† | — | 1200±65 |
| GPA | 2·74 (1·50–5·45) | 400±50 | 525±55 | 565±40 | 600±75 | 450±60 |
| UMP | — | 205† | 190* | 175* | — | 485† |

* Figure obtained from a single experiment.

† Average of two experiments.

present in the soluble fraction, but quantitative results on the amounts of these two substances there present were not sought.

The presence of PP in such considerable quantity was unexpected. The possibility was considered that this substance might have arisen as an artifact, from decomposition of ATP in the slightly alkaline solution from which the P compounds were adsorbed on the Dowex-1 resin, or by the resin itself. However, when it was found that the specific radioactivity of the PP was higher than that of the $P^{\gamma}$ of ATP in the 0·5 and 1 hr. experiments, it became evident that such an artifact could not account for the entire quantity of PP found. The possibility that some PP arose in this way cannot be rigorously excluded.

The outstanding quantitative differences between these results and those obtained by the less specific procedures used previously (Sacks, 1949) are the smaller amounts of PGA, G 1-P, and G 6-P found in the present experiments. The average values for these compounds are less than one-third as great as those obtained by differential hydrolysis procedures. Part of the discrepancies may be accounted for by species differences, but it is evident that the hydrolytic procedure employed in the previous work did not effect complete separation of AMP and UMP from G 6-P, so that the values for the amount of this substance reported earlier included considerable amounts of P from these nucleotides.

*Removal of tracer phosphate from plasma*

The results on the removal of tracer P from plasma, shown in Fig. 2, have been analysed in the same terms of half-times of the two processes and fractions of the total quantity of tracer accounted for by each, as was done previously (Sacks, 1953) for data obtained on rats. The half-time of the rapid process, representing principally exchange-adsorption with P of bone, was found to be about 15 min., compared with the 20 min. found in the rats, and the 20 min. calculated from the data of Sacks & Culbreth (1951) on cats. The half-time of the slow process, considered to represent principally the overall exchange rate with the P compounds of the soft tissues, was found to have the same value of about 3 hr. that was noted in the earlier experiments on rats and cats. The quantity of tracer P removed from the plasma by this slow process in the present experiments was only one-eighth of the injected amount, compared with one-fifth in the cats, one-third in one strain of rats, and one-fourth in the other strain of rats. The maximum values for the specific radioactivities of many of the intracellular P compounds, e.g. PP, ADP, ATP, were reached at 1 hr. after injection of the tracer, thus indicating high turnover rates for these substances.

In the experiments on rats (Sacks, 1951) it was found that the specific radioactivity of plasma P was equal to the specific radioactivity of the labile P of the ADP–ATP fraction at the time when the specific radioactivity for this intracellular fraction was at its maximum. This is the relation anticipated from the postulates of Zilversmit *et al.* (1943) if all the extracellular P is equally available for turnover with the entire quantity of those intracellular P compounds formed directly from extracellular P, or from intermediates so formed that are present only in trace amounts. When such a relation is found, i.e. when the curve for the time-course of the specific radioactivity of a precursor intersects that of the specific radioactivity of the product at the time of maximum specific radioactivity of the product, it is permissible to conclude that there is no 'compartmentalization' of the product substance within the cell. In the present experiments, this condition is not present; the specific radioactivity of plasma P is higher than

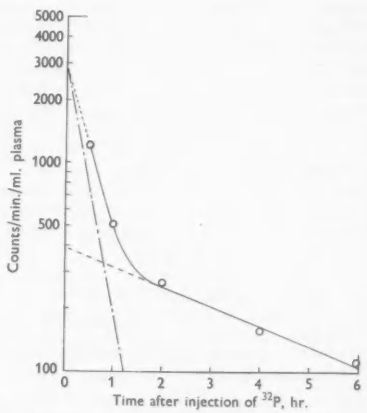


Fig. 2. Time-course of specific radioactivity of plasma P. Solid line, experimental data; short dashes, extrapolation of observed values to zero time; longer dashes, extrapolation of slow component to zero time; dot and dash line, calculated fast component of removal of tracer from plasma.

that of any of the intracellular P compounds for at least 2 hr. after the maximum specific radioactivities of the latter compounds has been reached. As the ultimate source of the $^{32}P$ for the labelling of the intracellular P compounds is plasma $^{32}P$, this finding implies either that during the first hour or so after the injection of the tracer, the specific radioactivity of the P in the extracellular phase available for turnover with intracellular compounds is considerably less than that of plasma P,

or that there is some 'compartmentalization', either structural or functional, of the intracellular P compounds. A third possibility is that both these factors apply.

The first of these alternatives implies that the transfer of phosphate ion across the capillary wall is a slow process, and does not take place by free diffusion, which would be rapid over the short distances involved. It is evident that the first few minutes after the intravenous injection of the tracer are required for mixing with the entire blood volume of the animal, and even if phosphate ion did diffuse freely across the capillary wall, some time would be required for equilibration of tracer P between plasma and extracellular phase of the tissues. However, the time factors in such processes are hardly long enough to explain the present results. Evidence for a slow, rather than a rapid, transcapillary movement of phosphate was obtained in the case of striated muscle (Sacks & Altshuler, 1942). In tracer experiments on cats, values were calculated for the specific radioactivity of the intracellular portion of the P, from the observed specific radioactivities and analytically determined amounts of plasma and muscle P, and the generally accepted values for the magnitude of the extracellular phase of muscle. These calculated values were negative for the first hour after injection of the tracer. The only acceptable alternative is that the passage of phosphate across the capillary wall into the interstitial fluid of the tissues is a slow process. Such an interpretation has been offered by Ennor & Rosenberg (1954) of their results on muscle. The other possibility they offer, that the extracellular phase of muscle is very much smaller than the measured 'chloride space' or 'sodium space', seems much less likely. The evidence for 'compartmentalization' is given below.

*Mechanism of transport of phosphate across cell membranes*

The measured values for the specific radioactivity of liver orthophosphate represent the mixture of intracellular orthophosphate present with both the interstitial fluid of the liver and the blood contained within the excised liver. It is impossible to arrive at a calculated value for the true intracellular orthophosphate of the liver which would have any significance, but it is evident that if free diffusion of phosphate ion took place across the cell membrane, isotopic equilibrium between orthophosphate of plasma and liver should be reached fairly rapidly. The observed values, showing great disparities between specific radioactivities of plasma and liver orthophosphate at 0·5 and 1 hr. after injection of the tracer, are not in keeping with the postulate of free diffusion. Furthermore, such a postulate would require a specific anion-permeability of the tissue cells for phosphate, in contrast to the well-established impermeability to smaller anions such as chloride, as well as requiring that the evidence for slow transfer of phosphate ion across the capillary wall be discarded. The observed values are in keeping with the postulate of active transport first proposed by Sacks & Altshuler (1942). The finding in the present experiments that the PP is the intracellular compound which acquires the highest specific radioactivity, and does so soon after the injection of the tracer, suggests strongly that the formation of this substance on the cell membrane is a major mechanism for the transport of orthophosphate into the cell from the extracellular phase.

*Mechanism for turnover of ATP*

The rapid attainment of isotopic equilibrium between PP and the two labile P groups of ATP, and the further finding that these two labile P groups have reached equal specific radioactivities at the earliest time of sampling after the injection of the tracer, suggest strongly the possibility that the principal mechanism for the formation of ATP in liver is the reaction of AMP with PP. This is in contrast with the report by Cobey & Handler (1953) that in muscle $P^{\beta}$ of ATP acquires the isotopic label much more slowly than $P^{\gamma}$. The breakdown of ATP into AMP and PP has been shown by Jones, Lipmann, Hilz & Lynen (1953) to furnish the energy for the acylation of coenzyme A, and is therefore an important step in the conversion of fatty acids into acetoacetic acid in the liver. A similar conversion of ATP into AMP and PP has been shown to take place in the activation of amino acids for protein synthesis in liver homogenates (Hoagland, 1955). The reversal of these two reactions, formation of ATP from AMP and PP, would account for the present results, and also allow for the conservation of the free energy of the PP bond.

The possible alternative mechanisms for the synthesis of ATP with the incorporation of $^{32}P$ are glycolysis, oxidative phosphorylation, and the myokinase reaction. If oxidative phosphorylation were to be an important mechanism for introducing $^{32}P$ into ATP, the intracellular orthophosphate of the liver would need to acquire a very high specific radioactivity shortly after the injection of the tracer. This could be achieved only by rapid and free diffusion of phosphate ion across the capillary wall and the liver cell membrane, both of which appear to be unlikely. The glycolytic pathway involves only $P^{\gamma}$ of ATP, and therefore is unable to account for the appearance of $^{32}P$ in $P^{\beta}$, much less to account for the rapid attainment of equality of specific radioactivity by $P^{\gamma}$ and $P^{\beta}$. Furthermore,

any $^{32}P$ acquired by $P^{\gamma}$ through the glycolytic pathway would be derived from G 1-P, G 6-P and PGA, and the specific radioactivity of these three compounds is considerably lower than that of $P^{\gamma}$ of ATP up to 1 hr. after injection of the tracer. These processes must be assumed to be operating, but their contribution to the incorporation of $^{32}P$ into ATP can hardly be significant in quantitative terms.

*Significance of the myokinase reaction*

Cobey & Handler (1953) obtained results which indicated a high apparent activity of myokinase in liver and kidney, but not in striated muscle. If the reaction catalysed by this enzyme, $2\,ADP \rightleftharpoons ATP + AMP$, were an important mechanism for the synthesis of ATP, then the specific radioactivity of the labile P of ADP should be at least equal to that of the two acid-labile P groups of ATP shortly after the injection of the tracer, and there should also be rapid attainment of equal specific radioactivities between AMP and the acid-stable P groups of ADP and ATP. Examination of Table 1 shows that neither of these is the case. The specific radioactivity of the acid-labile P of ADP increases more slowly than that of $P^{\beta}$ and $P^{\gamma}$ of ATP, and the AMP acquires the isotopic label at a much greater rate than do the acid-stable P groups of ADP and ATP. These results speak against any great importance of the myokinase reaction in the synthesis of ADP. However, the reversal of this reaction, the formation of ADP from AMP and ATP, is the most likely means of accounting for the presence of $^{32}P$ in the acid-labile P group of ADP. The energy for such a reversal could be derived from coupling with any of the oxidative stages in the metabolism of the liver.

*Mechanism of formation of AMP*

The results in Table 1 demonstrate that the rate of movement of the tracer into AMP is higher than that for the acid-stable P groups of ADP and ATP, and thereby rule out dephosphorylation of these two compounds as a principal mechanism for the formation of AMP. An alternative enzymic mechanism is available, namely the action of 5′-nucleotidase, which has been shown by Novikoff, Hecht, Podber & Ryan (1952) to be present in fairly high concentration in the cytoplasm of the liver cell. The operation of this enzyme in the direction of synthesis, again coupled with the energy supplied by oxidative reactions, could account for the observed findings. The P for this reaction could be derived from any of the intracellular reactions which liberate orthophosphate, e.g. ATPase, or from extracellular orthophosphate. The localization of this enzyme in the cell is such as to make extracellular orthophosphate an alternative possible source. The situation with respect to UMP is similar, with a much lower turnover rate.

*'Compartmentalization' of intracellular P compounds*

The localization of enzymes in different structural elements of liver, e.g. nuclei, mitochondria and microsomes, is well established. The 5′-nucleotidase referred to above is present to only a small extent in mitochondria (Novikoff, Podber & Ryan, 1950), while the major portion of the ATPase activity is present within the mitochondria (Novikoff *et al.* 1952; Kielley & Kielley, 1953). It is therefore quite possible that there is a functional 'compartmentalization' of the intracellular P compounds, in the sense that a considerable part of the ATP may be present in the cytoplasm, not attached to any of the enzymes in mitochondria for which it is the substrate. If such be the case, although much of the AMP is attached to 5′-nucleotidase in the cytoplasm, the high rate of turnover of AMP relative to that of the acid-stable P groups of ADP and ATP could be accounted for. A similar explanation is possible for the specific radioactivity of the plasma orthophosphate remaining above that of the intracellular orthophosphate, which in turn remains above that of the intracellular P compounds for so long after these have passed their maximum specific radioactivity.

*Origin of PGA*

The specific radioactivity of the PGA is significantly less than that reported in the previous studies (Sacks, 1951). The difference is probably due to the more specific method for the separation of this compound from others resulting from the use of the ion-exchange resin. The low specific radioactivities found here suggest that a considerable fraction of the PGA is derived by oxidation of α-GPA, rather than through the operation of glycolysis. It should be noted in this connexion that PGA is the only 3-carbon member of the glycolytic pathway which has been found in the intact liver cell in the absence of enzyme poisons. The α-GPA, on the other hand, is primarily an intermediate in phospholipid synthesis, present in some ten times the concentration of the PGA. The relations of specific radioactivity between PGA, α-GPA and ATP can be accounted for on the assumption that PGA in liver arises both through glycolysis and from the oxidation of α-GPA. This oxidation may be the first step in the degradation of the GPA arising from the turnover of liver phospholipids.

*Turnover of the sugar phosphates*

The relations between the specific radioactivities of G 1-P and ATP found in the present experiments are similar to those previously reported on rats (Sacks, 1951). However, the values for G 6-P, instead of being far below those of G 1-P as in the earlier experiments, are now intermediate between those of G 1-P and the labile P of ATP. This finding disposes of the discrepancy between the tracer findings *in vivo* with respect to the possible precursor of G 6-P and the known enzymic mechanisms for the formation of this substance. The earlier, erroneous, result was due to the inclusion in the G 6-P fraction of large amounts of P of low specific radioactivity from AMP and UMP. The present results are readily interpreted in terms of the simultaneous occurrence of the phosphoglucomutase and hexokinase reactions, without regard to whether there is net increase or decrease in the amount of liver glycogen. Although no glycogen determinations were made in the present experiments, it was clear that the amounts of glycogen precipitated by the addition of ethanol to the TCA extracts were much less in the 4 and 6 hr. experiments than in those of shorter duration. The specific radioactivity relations found with respect to G 1-P, G 6-P and ATP are compatible with a cyclic process for the interconversion of blood glucose and liver glycogen. Evidence suggesting the existence of such a cycle is obtained from the data of Stetten & Stetten (1954) on the time-course of the distribution of the isotope in the liver glycogen of rats fed on glucose labelled with $^{14}C$. In the first few hours the isotope was principally in the side branches of the glycogen molecule, but in rats killed more than 6 hr. after feeding the labelled sugar there was relatively more isotope in the central straight-chain portion of the glycogen than in the branches.

The specific methods used here for the isolation of G 1-P and G 6-P lead to the finding that these two compounds are present in the liver in approximately equal concentrations. This is quite different from the equilibrium in the phosphoglucomutase reaction, which was found by Colowick & Sutherland (1942) to be more than 90% on the side of G 6-P. The conditions of the present experiments are reasonably close to those of the physiological steady state with respect to these two compounds; it is therefore apparent that factors other than the purely thermodynamic ones operating on the isolated enzyme system govern the relative concentrations in the intact cell.

The high turnover rate of the P of PHCA is in keeping with that reported by Umbreit (1953) in rat liver. However, it is difficult to correlate the present data with the low turnover found by Umbreit for the fraction of 'ester P', since this probably represents a mixture of compounds of varying turnover rates.

The few results it was possible to obtain on the turnover rate of F 1-P are inadequate to establish a precursor for this substance. If the mechanism involved is the transfer of P from ATP to fructose, as postulated by Leuthardt, Testa & Wolf (1953), then it would appear that this reaction is a slow one. The specific activity of the F 1-P in the 6 hr. experiments, for which complete information is available, indicates that isotopic equilibrium with $P^{\gamma}$ of ATP has not yet been reached. Such a slow turnover is not unexpected, since no fructose was entering the liver.

The nature of the not easily hydrolysable P compound eluted from the resin together with PP has not been investigated. One possibility is that it is the 2:3-diPGA which Rapoport & Guest (1941) found to be present in rather large quantity in mammalian erythrocytes. The properties of this compound are such that it would be expected to follow PP closely in the elution from the resin. Assuming this composition for the compound found, the question remains open whether it is present intracellularly in liver or is merely a contaminant resulting from the presence of quantities of erythrocytes in the liver as excised. This question can be answered by comparing the specific radioactivities of the compound isolated from liver and erythrocytes.

## SUMMARY

1. A time-course study has been made of the distribution of tracer phosphate between plasma P and the acid-soluble P compounds of rabbit liver. Ion-exchange chromatography has been used as the principal means of isolating the various P compounds.

2. The removal of the tracer has been found to follow the same biphasic exponential function as previously found in other mammalian species; the half-times of the two processes were found to be about 15 min. and 3 hr.

3. The slow process, which is considered to represent principally the exchange with the P compounds of the soft tissues, accounts for about one-eighth of the tracer removed from the plasma.

4. The results obtained suggest that there is a functional, if not a structural, 'compartmentalization' of some of the acid-soluble P compounds within the cell, so that the entire quantity of some of them may not be equally available for turnover processes.

5. Inorganic pyrophosphate has been demonstrated to be present in the liver and to have a higher turnover rate than any of the intracellular organic P compounds. The results are consistent

with the hypothesis that the formation of pyrophosphate on the cell membrane serves as a principal means for the entry of phosphate into the cell interior.

6. The results obtained also indicate that adenosine monophosphate (AMP) and uridine 5′-phosphate (UMP) may be formed by reactions at the cell membrane.

7. The principal mechanism for the formation of adenosine triphosphate (ATP) is indicated to be the reaction of AMP with pyrophosphate. The myokinase reaction and the glycolytic pathway appear to be of secondary quantitative importance in this respect.

8. The principal mechanism for the formation of adenosine diphosphate appears to be the reversal of the myokinase reaction.

9. The concentrations of glucose 1-phosphate (G 1-P) and glucose 6-phosphate (G 6-P) in the liver were found to be approximately equal, rather than with the G 6-P predominating, as required by the equilibrium of the phosphoglucomutase reaction.

10. The specific radioactivity relations between G 1-P, G 6-P and $P^{\gamma}$ of ATP are such as to indicate that G 6-P is formed by the phosphoglucomutase and hexokinase reactions taking place simultaneously. This suggests that there is a continuous cycling between blood glucose and liver glycogen whether or not there is net deposition of glycogen in the liver.

11. The specific radioactivity relations of α-glycerophosphate (GPA), phosphoglyceric acid (PGA) and $P^{\gamma}$ of ATP suggest that the oxidation of GPA rather than the glycolytic pathway is the principal means of formation of PGA.

12. The turnover rate of fructose 1-phosphate was found to be considerably lower than that of G 1-P.

13. The turnover rate of UMP is considerably lower than that of AMP.

14. The evidence suggests that the principal means for the formation of AMP and UMP is the action of 5′-nucleotidase, operating in the direction of synthesis by coupling with oxidative reactions.

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# Amino Acid Sequence in Lysozyme

## 2. ELUTION CHROMATOGRAPHY OF PEPTIDES ON ION-EXCHANGE RESINS*

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The previous paper in this series (Thompson, 1955) reported some amino acid sequences in lysozyme which were deduced from the structures of a number of small peptides isolated from partial acid hydrolysates by displacement chromatography on ion-exchange resins and further fractionation on paper chromatograms. Despite the success achieved by Partridge and co-workers (see Partridge & Brimley, 1952) in the separation of amino acids by displacement chromatography and the high capacity of such columns, it was apparent in these experiments that the relatively non-specific van der Waals interactions between the larger peptide molecules and the resin outweighed the effect of differences in the p$K$ values of their ionizing groups, with the result that many peptides were similarly absorbed and thus not differentiated. Nevertheless, a large number of peptides were isolated and identified in these experiments.

Experiments using elution chromatography on ion-exchange resins for the preliminary separation of peptides from an acid hydrolysate of lysozyme are described in this paper. The high resolving power of this method for amino acids was elegantly demonstrated by Moore & Stein (1951). Dowmont & Fruton (1952) have shown that a number of simple peptides may be satisfactorily chromatographed in this way. Schroeder, Kay, Le Gette, Honnen & Green (1954) have chromatographed a partial acid hydrolysate of gelatin in Dowex-50 × 8 and examined the material in the eluate. After further resolution of the peptides as $N$-2:4-dinitrophenyl (DNP) derivatives on silicic acid–Celite columns a number of di- and tri-peptides were identified in gelatin hydrolysates. A similar examination was made of silk-fibroin hydrolysates by Kay & Schroeder (1954). This procedure has the advantage that no desalting step is necessary to remove buffer salts from the eluant fractions.

The present experiments have borne out the expectation that the elution procedure would possess the highest general resolving power of any method yet developed for the separation of peptides. However, the complexity of a partial acid hydrolysate of a protein is such that it has not been possible to obtain pure peptides by this method alone. Nevertheless, the fractions representing peaks in the elution curve are fairly simple and can readily be further fractionated into pure peptides by two-dimensional paper chromatography. The identification of most of these peptides from an acid hydrolysate in this manner has allowed the compilation of further sequences in lysozyme.

Sulphonated polystyrene (Dowex-50) crosslinked with 4% divinylbenzene (cf. Dowmont & Fruton, 1952) has been used in the present experiments. Successful chromatography is achieved with this degree of crosslinkage, which allows penetration of peptides containing up to four or five residues, whereas the 8% crosslinked resin used for amino acid analysis is unsuitable (Moore & Stein, 1951).

### METHODS

*Preparation of hydrolysate of lysozyme.* The egg-white lysozyme (Armour and Co.) used in these experiments was the same as that used previously (Thompson, 1955). Lysozyme (500 mg.) was hydrolysed at 37° for 4 days with 12N-HCl and treated as previously with performic acid to oxidize the disulphide bonds and convert the half-cystine residues into cysteic acid (Thompson, 1955). Methionine residues are converted into 'methionine sulphone' residues by this treatment.

*Chromatography of lysozyme hydrolysate.* The elution chromatogram described in this paper was run with gradient elution with ammonium formate and acetate buffers which were sublimed to give salt-free fractions (cf. Hirs, Moore & Stein, 1952). However, this procedure necessitated removal of ammonia from the samples of solution used to plot the elution peaks, since ammonia, like the amino acids and peptides, gives a ninhydrin colour. Alternatively, the chromatogram could have been run with sodium salts, when the elution curve could be plotted without preliminary treatment. However, each fraction would then have required separate desalting treatment (cf. Stein, 1953), and rather large columns would have been needed to handle the large volumes of effluent buffer in the less sharply defined peaks. The sodium cycle would also have necessitated individual neutralization of each eluate

* Part 1: Thompson (1955).

fraction in plotting the effluent peaks, or development of a ninhydrin reagent with sufficient buffering power to overcome this tedious operation (S. Moore & W. H. Stein, private communication).

A sample of sulphonated-polystyrene cation-exchange resin (Dowex-50, supplied by Dow Chemical Company) crosslinked with 4% divinylbenzene and graded 200–400 mesh/in. was first passed through a 40 mesh/in. sieve to remove coarse particles. In order to give a satisfactory flow rate in the column, it was found necessary to remove with a 270 mesh/in. sieve a large amount of fine material which otherwise reduced the flow rate seriously. The resin was prepared for chromatography according to the procedure of Hirs *et al.* (1952), the final wash being with 0·05 M ammonium formate buffer (pH 2·49) (molarity refers to cation, see Table 1) until the pH remained constant.

A column of diameter 1·8 cm. and height 2 m. was packed in portions as described by Moore & Stein (1951), but without applying pressure. The column was again washed with 0·05 M ammonium formate (pH 2·49) till the effluent pH was the same as that of the solution entering the column.

The column was set up for gradient elution by inserting a buffer mixer in the line between the reservoir funnel and the top of the column. This consisted of a 500 ml. flask with ground-glass joint carrying an inlet tube, connected by Tygon tubing (Arthur H. Thomas, Philadelphia 5, Pa., U.S.A.) to the reservoir, reaching half-way down the flask, and an outlet to the top of the column through Tygon tubing. The flask was stirred continuously by a magnetic stirrer. Thus discontinuous buffer changes resulted in smoothly continuous increase in the pH and ionic strength of the solution admitted to the top of the column.

The hydrolysate was taken up in the first buffer (10 ml.; pH 2·49), added to the top of the column, and washed in with a further 10 ml. of buffer. A certain amount of material remained as a sludge and was also transferred. The buffer mixer, connecting tubes and top of the column were entirely filled with buffer, which was allowed to flow down the column at a rate of 8 ml./hr. Fractions of 8 ml. were collected with a Technicon fraction collector (Technicon Company, 215 E. 149th Street, New York 51) on a drop-counting basis. A preliminary experiment had indicated approximately the points at which changes of buffer should be made. The composition of the buffers used is given in Table 1.

The peptide and amino acid material in the tubes was located by taking a 0·2 ml. sample from every second tube and determining the colour yield with ninhydrin, according to the procedure of Moore & Stein (1948), 2 ml. ninhydrin solution and 10 ml. diluent being used.

Table 1. *Composition of buffers*

Based in part on the buffers used by Hirs, Moore & Stein (1952). 1·5% (v/v) of benzyl alcohol was added to all buffers.

| | | Buffer composition | | | |
|---|---|---|---|---|---|
| | | | Vol. of 2 M acid | Vol. of M-$NH_3$ | Vol. of buffer used in elution run (ml.) |
| | | | To final vol. 1 l. with water | | |
| Molarity of $NH_4^+$ | pH | Anion | | | |
| 0·05 | 2·49 | Formate | 450 | 50 | * |
| 0·2 | 3·44 | Formate | 263 | 200 | 1500 |
| 0·2 | 3·67 | Formate | 200 | 200 | 1500 |
| 0·2 | 4·04 | Formate | 138 | 200 | 2000 |
| 0·2 | 4·28 | Formate acetate | † | † | 1500 |
| 0·2 | 4·50 | Acetate | 220 | 200 | 1500 |
| 0·4 | 4·93 | Acetate | 300 | 400 | 1000 |
| 0·4 | 5·11 | Acetate | 500 | 400 | 1500 |
| 0·6 | 5·62 | Acetate | 400 | 600 | 1500 |
| 0·6 | 6·27 | Acetate | 385 | 600 | 1000 |
| 0·6 | 6·81 | Acetate | 370 | 600 | 1000 |

* This buffer was used to equilibrate the column, add the hydrolysate to the column and fill the mixer reservoir and connecting tubing (approx. 600 ml.), and the next buffer (pH 3·44) was added to the separating funnel.

† A mixture of ammonium formate buffer (pH 4·04) (2 vol.) and ammonium acetate buffer (pH 4·50) (1 vol.).

As ammonia itself gives a colour with ninhydrin, the ammonium acetate from the buffer was first removed by evaporating the sample to dryness overnight at 37° in an evacuated desiccator over NaOH and $H_2SO_4$, adding 0·5 ml. N-NaOH to the sample and re-evaporating overnight as before, and finally adding a drop of water to each tube and evaporating to dryness again over fresh $H_2SO_4$. This last step was necessary as the sulphuric acid formed an ether- and acetone-soluble and water-insoluble scum, presumably a polymerized derivative of formic and acetic acids. This prevented the ammonia being completely absorbed and led to some variability in the results. The NaOH was neutralized with 1 ml. 0·5 N-HCl before the addition of the ninhydrin solution. The tubes were heated in a boiling-water bath for 30 min. instead of 20 min. (since water boils at 93° at Salt Lake City and since the rate of reaction of the complex mixture of peptides with ninhydrin is not known).

An elution curve (Fig. 1) was obtained by plotting the colour density against the fraction number. Approximately fifty more-or-less sharply defined peaks were obtained and the eluate was divided into a corresponding number of fractions by combining the contents of the tubes under each peak.

Each fraction was evaporated to dryness and the material finally distributed as a film on the walls of a large

flask. The ammonium acetate was removed by sublimation on a cold-finger water condenser under high vacuum overnight, a trap cooled in solid $CO_2$–ethanol being inserted before the pump. Solution and redistribution on the walls of the flask and resublimation gave efficient removal of virtually all the ammonium acetate.

The fractions were examined as previously described (Thompson, 1955). Two-dimensional chromatography was used to isolate the individual peptides, which were eluted after detection with dilute ninhydrin. A portion of each peptide fraction was hydrolysed to determine the constituent amino acids. The *N*-terminal end group was determined by the DNP method (Sanger & Thompson, 1953*a*). In suitable cases, when more material was available, carboxypeptidase was used to determine the *C*-terminal residue. As in the previous paper (Thompson, 1955), evidence for such a *C*-terminal amino acid is denoted by italicizing this residue. In a limited number of cases partial acid hydrolysis of the DNP-peptide was used to establish the sequence (cf. Sanger, 1949). The DNP-peptide was partially hydrolysed with 12 N-HCl for 6 days at 37°. If the colour could not be extracted into ether or ethyl acetate, the DNP-peptide was freed from contaminating peptides and amino acids by adsorption on a column of talc (see Sanger, 1949). This step was necessary, otherwise the amino acids and peptides overlapped with the DNP derivatives. The latter were separated from each other on a paper chromatogram run with the *tert.*-pentanol

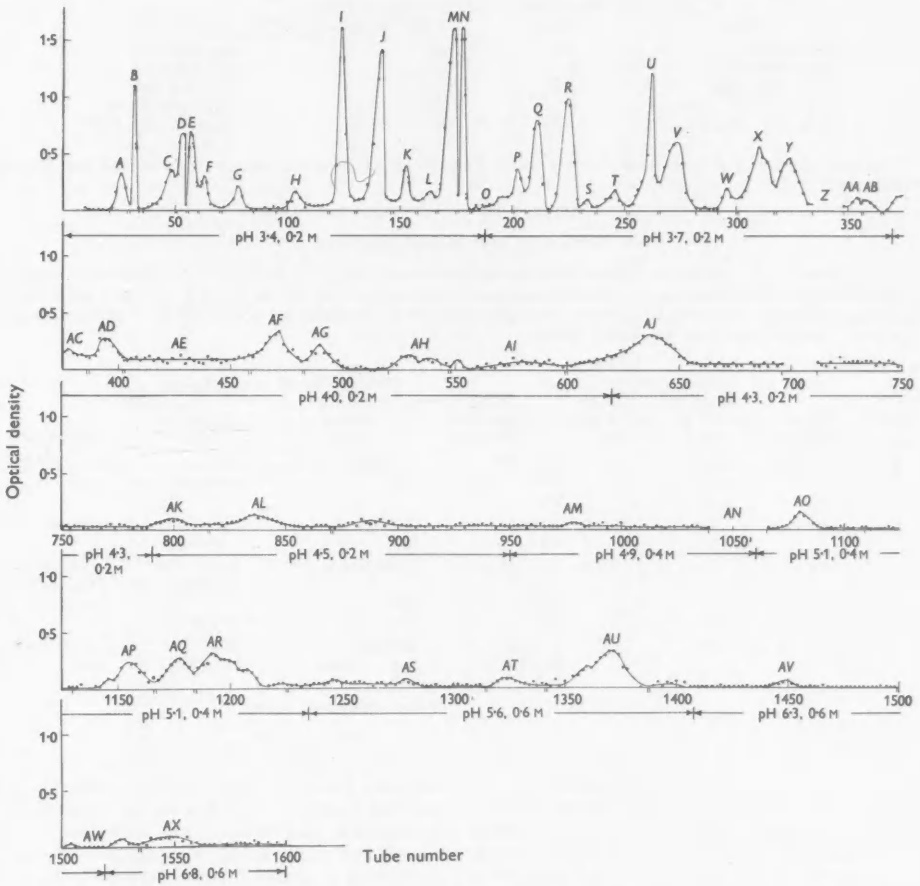


Fig. 1. Chromatogram of a partial acid hydrolysate of lysozyme on sulphonated-polystyrene resin (Dowex-50 × 4) eluted with ammonium formate and acetate buffers (Table 1). Gradient elution was used and the pH and molarity of the buffers are indicated below the curve. The vertical lines below the abscissa indicate the tubes which were combined to give fractions denoted by the letters above the curve. For other details see text.

(2-methylbutan-2-ol)–pH 6 buffer system used for DNP-amino acids. The DNP-peptide spots were eluted with acetone–N-HCl (1:1, by vol.), hydrolysed and the constituent amino acids examined on paper chromatograms. Identification of a DNP-dipeptide established the sequence of the first two residues in the original peptide and, in the case of a tripeptide, the complete sequence.

## RESULTS

The results where the partial hydrolysis of DNP-peptides was studied are given in Table 2.

tion contained about eight components which the subsequent two-dimensional chromatogram was capable, in most instances, of resolving into pure peptides. The neutral amino acids emerged early (with leucine in fraction *U*) and in the same order as described by Moore & Stein (1951). The peptides in general were retarded somewhat in comparison (e.g. whereas threonine appears in fraction *J* and alanine in fraction *N*, Thr.Ala emerges in fraction *X*). The basic amino acids lysine and histidine occurred in fraction *AL* and arginine in fraction

Table 2. *Sequences from partial acid hydrolysis of DNP-peptides*

| Peptide | Fraction (Fig. 1 and Table 4) | Amino acid identified in DNP-peptide | Sequence |
|---|---|---|---|
| Ser.(Asp, Arg) | *AP* | Asp | Ser.Asp.Arg |
| Thr.(Asp, Gly) | *R* | Gly | Thr.Gly.Asp |
| Thr.(Asp, Arg) | *AQ* | Asp | Thr.Asp.Arg |
| Gly.($CySO_3H$, Asp) | *C* | $CySO_3H$ | Gly.$CySO_3H$.Asp |
| Leu.(Gly, Ala, Val) | *AE* | Gly + + + + + + | Leu.Gly.Ala.Val* |
| | | Ala + + | |

* The large amount of glycine and the lesser amount of alanine are taken to indicate a mixture of DNP-Leu.Gly and DNP-Leu.Gly.Ala.

Table 3. *Peptides from fraction C*

For the isolation of peptides, two-dimensional chromatograms were run with phenol in an atmosphere of ammonia (0·3%) in the first direction and with a butanol–acetic acid–water mixture (40:10:50, by vol.) (aged at least 3 days) in the second dimension. Whatman no. 3 paper, chromatographically washed with 2N acetic acid followed by water, was employed. Relative intensities of spots are indicated roughly by + signs.

| Spot no. | Spot intensity | Distance moved in phenol–$NH_3$ (cm.) | Distance moved in butanol–acetic acid (cm.) | Amino acids after hydrolysis | DNP treatment and hydrolysis: Amino acids | DNP-amino acids | Structure |
|---|---|---|---|---|---|---|---|
| 1 | + + + + | 3·5 | 8 | $CySO_3H$ + + + | $CySO_3H$ + + | Gly | Gly.($CySO_3H$, |
| | | | | Asp + + + | Asp + + | (medium) | Asp) |
| | | | | Gly + + + | | | |
| 2 | + + + | 0·5 | 8 | $CySO_3H$ + + + | $CySO_3H$ trace | $CySO_3H$† | $CySO_3H$.Asp |
| | | | | Asp + + + | Asp + + | (weak) | |
| 3 | + + + | 32·5 | 12 | $CySO_3H$ + + + | $CySO_3H$ + + + | Asp | Asp.($CySO_3H$, |
| | | | | Asp + + + | | (medium) | Pro, Ileu) |
| | | | | Ileu + + + | Ileu + + + | | |
| | | | | Pro * | Pro * | | |
| 4 | + | 23 | 17 | $CySO_3H$ + + | $CySO_3H$ + + | Asp | Asp.($CySO_3H$, |
| | | | | Asp + + | Asp trace | (medium) | Leu) |
| | | | | Leu + + | Leu + + | | |

* Yellow spot. † Yellow aqueous layer.

The results of the examination of the peptides in a typical fraction (*C*) are given in Table 3.

Table 4 shows the peptides contained in each fraction, where they were obtained pure and the results of the determination of their structure were clear-cut.

Although fairly sharp, symmetrical peaks were obtained with a number of fractions, it was clear from subsequent analysis that each fraction, corresponding to one peak, did not represent a pure amino acid or peptide. On an average, each fraction contained about eight components which the subsequent two-dimensional chromatogram was capable, in most instances, of resolving into pure peptides. The neutral amino acids emerged early (with leucine in fraction *U*) and in the same order as described by Moore & Stein (1951). The peptides in general were retarded somewhat in comparison (e.g. whereas threonine appears in fraction *J* and alanine in fraction *N*, Thr.Ala emerges in fraction *X*). The basic amino acids lysine and histidine occurred in fraction *AL* and arginine in fraction *AR*. The positions of the peptides in the elution sequence relative to each other correspond closely to what would be expected on the basis of their composition and the elution behaviour of the constituent amino acids. For instance, dipeptides containing *N*-terminal arginine linked with, as *C*-terminal residue, cysteic acid, aspartic acid, glycine or leucine, emerge in fractions *AF*, *AO* (and *AQ*), *AV* and *AX* respectively, in the order of emergence of these amino acids when free.

Table 4. *Peptides in elution fractions*

The major component of each fraction is indicated by an asterisk, and free amino acids are printed in capital letters. Where the sequence is unknown the amino acids are enclosed within brackets.

| Fraction | Components | Fraction | Components |
|---|---|---|---|
| *A* | Artifact | *S* | — |
| *B* | CYSTEIC ACID* | *T* | ISOLEUCINE* |
| *C* | Gly.($CySO_3H$, Asp)* | | Ala.$MetO_2$ |
| | $CySO_3H$.Asp | | Gly.$MetO_2$* |
| | Asp.($CySO_3H$, Leu) | | Asp.(Ala, $MetO_2$) |
| | Asp.($CySO_3H$, Pro, Ileu) | | (Asp.Ser) |
| *D* | $CySO_3H$.Ala* | *U* | LEUCINE* |
| | $CySO_3H$.Glu | | Ala.$MetO_2$ |
| | $CySO_3H$.(Glu, Ala) | | Thr.(Asp, Gly) |
| | $CySO_3H$.(Asp, Gly) | | Ser.(Asp, Gly) |
| *E* | Ser.($CySO_3H$, Asp, Val)* | | $MetO_2$.Asp |
| | Asp.($CySO_3H$, Pro, Ileu)* | | (Asp, Leu) |
| | Val.$CySO_3H$ | | Asp.Ala |
| | ($CySO_3H$, Glu) | | (Asp, Glu, Ala, Val) |
| | ($CySO_3H$, Ala) | | (Asp, Glu, Val) |
| | Val.($CySO_3H$, Ala) | *V* | Thr.Glu* |
| *F* | $CySO_3H$.(Glu, Leu)* | | (Glu, Thr, Ala) |
| | Asp.($CySO_3H$, Leu)* | | (Asp, Ser, Val) |
| | Leu.$CySO_3H$ | | Ala.$MetO_2$ |
| | Asp.($CySO_3H$, Ileu) | | Ser.(Asp, Ileu) |
| | ($CySO_3H$, Asp) | | Ser.(Asp, Gly) |
| | Leu.($CySO_3H$, Asp) | | (Asp, Glu, Ala, Val) |
| | $CySO_3H$.Ileu | | Asp.Leu |
| *G* | Asp.($CySO_3H$, Ileu) | *W* | (Asp, Glu, Ileu)* |
| *H* | Asp.Asp* | | (Asp, Ileu) |
| *I* | ASPARTIC ACID* | *X* | Glu.(Asp, Ileu)* |
| *J* | SERINE* | | Asp.Ileu* |
| | THREONINE | | Thr.Ala* |
| | METHIONINE SULPHONE | | Glu.Ala |
| *K* | GLUTAMIC ACID* | | (Asp, Ala, Ileu) |
| | PROLINE | | Ser.Ala |
| | SERINE (trace) | | Glu.(Asp, Ala) |
| | (Asp, Gly) | *Y* | Ala.Ala* |
| *L* | GLUTAMIC ACID (small amount)* | | (Asp, Ileu) |
| | Asp.Ileu* | | Thr.(Asp, Glu, Ala, Val) |
| | Asp.Gly | | Val.(Glu, Ala) |
| *M* | GLYCINE* | | Asp.(Gly, $MetO_2$) |
| | ALANINE | | Ser.(Asp, Val) |
| | (Asp, Ileu) | | (Asp, Ala, Val) |
| *N* | ALANINE* | | (Ser, Ala) |
| | GLYCINE | | (Asp, Glu, Ala, Val) |
| | Val.($CySO_3H$, Asp, Ala) | *AA* | (Pro, Gly) |
| | (Asp, Leu) | | Glu.(Asp, Ileu) |
| *O* | GLYCINE* | | Val.Glu |
| | ALANINE | *AB* | (Pro, Gly)* |
| | Asp.Gly | | Glu.(Asp, Ileu)* |
| | VALINE | | Val.Glu |
| *P* | (Asp, Gly)* | *AC* | Glu.(Ala, Leu)* |
| | (Asp, Glu, Val) | | $CySO_3H$.Lys* |
| *Q* | Thr.Asp* | | Ser.Val |
| | (Asp, Ser, Gly) | | (Asp, Ileu) |
| | (Asp, Glu, Ala) | *AD* | Thr.(Gly, Pro)* |
| *R* | Ser.(Asp, Gly)* | | Val.Ala |
| | Thr.(Asp, Gly) | | Val.(Thr, Gly, Pro, Ala) |
| | Asp.Ala | | Thr.(Glu, Gly, Ala) |
| | Thr.(Asp, Gly, Val) | | $CySO_3H$.(Gly, Lys) |
| | Asp.(Val, Glu) | *AE* | Glu.(Ala, Leu, Ileu)* |
| | Ser.Asp | | (Ala, Leu)* |
| | Val.Asp | | Leu.(Gly, Ala, Val)* |
| | | | ($CySO_3H$, Gly, Lys) |

* For note see p. 258.

Table 4 (*cont.*)

| Fraction | Components | Fraction | Components |
|---|---|---|---|
| *AF* | Glu.Leu*<br>Gly.Leu*<br>Ileu.(Ser, Gly, Leu)*<br>Arg.$CySO_3H$<br>Ser.(Asp, Ileu)??<br>Ileu.(Glu, Leu) | *AL* | LYSINE*<br>HISTIDINE<br>(His, Lys)<br>Ser.($CySO_3H$, Asp, Leu, Arg)<br>Ser.(Asp, Thr, Ala, Ileu)?<br>Asp.(Thr.Ala, Ileu) |
| *AG* | Ser.Leu*<br>Ala.Leu* | *AM* | Asp.Arg* |
| | Ala.(Glu, Ileu, Leu)<br>$CySO_3H$.Lys<br>(Arg, $CySO_3H$, Glu)<br>(Ser, Val) | *AO* | Arg.Asp*<br>Ala.Lys |
| *AH* | Phe.Asp* | *AP* | Ala.Lys*<br>Ala.(Gly, Lys)* |
| | $CySO_3H$.Arg<br>Asp.Arg<br>($CySO_3H$, Glu, Ala, Leu)<br>(Tyr, Asp)<br>Gly.($CySO_3H$, Arg)<br>Ileu.Pro | | Ser.(Asp, Arg)<br>Ileu.(Thr, Ala) |
| *AI* | $CySO_3H$.(Asp, Gly, Arg)<br>(Asp, Arg) | *AQ* | Lys.Gly*<br>Ala.($MetO_2$, Lys)<br>Arg.Asp<br>Thr.(Asp, Arg) |
| *AJ* | Phe.Glu* | *AR* | ARGININE*<br>(Asp, Arg) |
| | Phe.Asp<br>(Glu, Phe, Gly)<br>Ser.(Asp, Phe)<br>$MetO_2$.($CySO_3H$, Lys)<br>Asp.(Gly, Arg)<br>(Asp, Arg) | *AS* | $MetO_2$.Lys* |
| | | *AT* | Val.(Ileu, Lys)*<br>$MetO_2$.($CySO_3H$, Lys, Arg) |
| | | *AU* | Ser.Arg*<br>(Asp, Thr, Arg) |
| *AK* | $CySO_3H$.(Arg, Leu)* | *AV* | Lys.(Phe, Glu)* |
| | Phe.(Asp, Glu)*<br>(Asp, Ser, Phe)*<br>Asp.($CySO_3H$, Leu, Arg)?<br>(Asp, Arg)<br>Asp.(Gly, Arg)<br>Ser.($CySO_3H$, Asp, Leu, Arg)<br>($CySO_3H$, Asp, Leu, Arg) | | (Arg.Gly)<br>($CySO_3H$, Lys, Arg) |
| | | *AW* | Ileu.Arg*<br>Arg.($CySO_3H$, Gly, Lys) |
| | | *AX* | Arg.Leu*<br>(Arg, Ileu) |

*Note.* $R_F$ values of the peptides were not obtainable as the solvent was allowed to drip off the bottom of the paper; the chief aim of the experiment was the isolation of pure peptides rather than their characterization by $R_F$ values. Thus only the distances moved by the peptides could be recorded. The peptides listed above were isolated from a number of different chromatograms and a comparison of distances moved by peptides on different chromatograms would be misleading. No common reference substance was included on chromatograms.

In general, any particular peptide did not spread over more than three consecutive fractions. For example, the peptide Ala.$MetO_2$ was identified in fractions *T*, *U* and *V*. This is to be expected on theoretical grounds if a peak does not return to the base-line on either side and the fraction is cut at the positions of minimum ninhydrin colour. This is true even when the substance is responsible for the shape of the elution peak; the position of the fraction cuts is even more arbitrary when a superimposed substance, present in higher concentration, determines the position of the cuts. In this particular instance free leucine, largely responsible for peak *U*, determined the cutting. In a few instances spreading over more than three fractions was observed. For example, peptides containing aspartic acid and isoleucine were found in fractions *L*, *M*, *W*, *X*, *Y* and *AC*. It was not always practicable to determine the *N*-terminal residue on every such peptide. Hence some doubt exists as to whether all the peptides have the same sequence. Furthermore, with peptides containing aspartic and glutamic acids, uncertainty exists as to whether the presence of this amino acid in its amide form, owing to incomplete removal of the ammonia during partial acid hydrolysis, introduces a second species in the chromatogram. In this particular case it would certainly appear that the compound appearing in fractions *L* and *M* is different from that occurring in the later fractions which are widely separated from *L* and *M*.

In Table 5 are listed all the peptides that have been isolated from lysozyme in this work and

*Note on Table 5 (opposite).* In the preliminary elution experiment a few peptides were isolated which were not encountered in the main experiment. They are also listed as occurring in the corresponding fraction of the main experiment but are marked.†

Table 5. *Peptides from acid hydrolysis of lysozyme*

* Indicates that the peptide was isolated after displacement chromatography (Thompson, 1955).

| Peptide | Fraction |
|---|---|
| Ala.Ala | *Y* |
| *Ala.Leu | |
| Ala.Lys | *AO, AP* |
| *Ala.$MetO_2$ | *U, V* |
| Ala.(Gly, Lys) | *AP* |
| Ala.($MetO_2$, Lys) | *AQ* |
| Ala.(Glu, Ileu, Leu) | *AG* |
| *Ala.(Asp, Glu, Ala?) | |
| *Arg.Asp | *AO, AQ* |
| *Arg.$CySO_3H$ | *AF* |
| Arg.Gly | *AV* |
| Arg.Leu | *AX* |
| *Arg.($CySO_3H$, Asp) | |
| *Arg.($CySO_3H$, Glu) | *AG* |
| *Arg.($CySO_3H$, Lys) | *AV* |
| Arg.($CySO_3H$, Gly, Lys) | *AW* |
| *Asp.Ala | *R, U* |
| *Asp.Arg | *AH, AK, AM* |
| Asp.Asp | *H* |
| *Asp.Gly | *L, O* |
| *Asp.Ileu | *L, X* |
| Asp.Leu | *V* |
| Asp.(Ala, $MetO_2$) | *T* |
| Asp.($CySO_3H$, Ileu) | *F, G* |
| Asp.($CySO_3H$, Leu) | *C, F* |
| Asp.($CySO_3H$, Arg) | *N* |
| Asp.(Gly, Arg) | *AJ, AK* |
| *Asp.(Gly, $MetO_2$) | *Y* |
| *Asp.(Glu, Ala) | |
| *Asp.(Glu, Ileu) | |
| *Asp.(Glu, Val) | *R* |
| Asp.($CySO_3H$, Leu, Arg) | *AK* |
| Asp.($CySO_3H$, Pro, Ileu) | *C, E* |
| Asp.(Gly, Leu, Arg) | *AJ* |
| *Asp.(Glu, Ala, Val) | |
| Asp.(Thr, Ala, Ileu) | *AL* |
| $CySO_3H$.Ala | *D* |
| $CySO_3H$.Arg | †*AH* |
| $CySO_3H$.Asp | *C* |
| $CySO_3H$.Glu | *D* |
| $CySO_3H$.Ileu | *F* |
| $CySO_3H$.Lys | *AC* |
| *$CySO_3H$.(Ala, Lys) | |
| $CySO_3H$.(Arg, Leu) | *AK* |
| $CySO_3H$.(Asp, Gly) | *D* |
| *$CySO_3H$.(Gly, Lys) | *AD, AE* |
| $CySO_3H$.(Glu, Ala) | *D* |
| $CySO_3H$.(Asp, Gly, Arg) | *AI* |
| Gly.Leu | *AF* |
| Gly.$MetO_2$ | †*T* |
| Gly.($CySO_3H$, Arg) | *AH* |
| Gly.($CySO_3H$, Asp) | *C* |
| *Glu.Ala | *X* |
| *Glu.Leu | *AF* |
| *Glu.(Ala, Leu) | *AC* |
| Glu.(Asp, Ala) | *X* |
| *Glu.(Asp, Ileu) | *X* |
| Ileu.Arg | *AW* |
| *Ileu.Asp | |
| Ileu.Pro | *AH* |
| Ileu.Val | †*AH* |
| Ileu.(Asp, Ala) | †*Y* |
| Ileu.(Glu, Leu) | *AF* |
| Ileu.(Thr, Ala) | *AP* |
| Ileu.(Ser, Gly, Leu) | *AF* |
| *Leu.Ala | *AG* |
| *Leu or Ileu.Glu | |
| Leu.$CySO_3H$ | *F* |
| Leu.Leu | †*AJ* |
| Leu.($CySO_3H$, Asp) | *F* |
| *Leu.(Thr, Ala) | |
| Lys.Gly | *AQ* |
| Lys.(Phe, Glu) | *AV* |
| $MetO_2$.Asp | *U* |
| $MetO_2$.Lys | *AS* |
| $MetO_2$.($CySO_3H$, Lys) | *AJ* |
| $MetO_2$.($CySO_3H$, Lys, Arg) | *AT* |
| Phe.Asp | *AH* |
| Phe.Glu | *AJ* |
| *Phe.(Asp, Glu) | *AK* |
| *Ser.Ala | *Y* |
| Ser.Arg | *AU* |
| *Ser.Asp | *R* |
| *Ser.Leu | *AG* |
| Ser.Val | *AC* |
| Ser.(Asp, Arg) | *AP* |
| *Ser.(Asp, Gly) | *R, U, V* |
| Ser.(Asp, Ileu) | *V, AF* |
| *Ser.(Asp, Leu) | |
| Ser.(Asp, Phe) | *AJ* |
| *Ser.(Asp, Val) | *Y* |
| Ser.(Gly, Ala) | *X* |
| *Ser.(Gly, Leu) | †*AG* |
| *Ser.(Leu, Arg) | |
| *Ser.(Asp, Gly, Ala) | |
| *Ser.(Asp, Gly, $MetO_2$) | |
| *Ser.($CySO_3H$, Asp, Val) | *E* |
| Ser.($CySO_3H$, Ala, Val, Lys) | *AF* |
| Ser.($CySO_3H$, Asp, Leu, Arg) | *AK, AL* |
| *Ser.(Gly, Ala, Leu?, *Leu*) | |
| Ser.(Asp, Glu, Ala, Ileu, Leu) | *AG* |
| Leu.(Gly, Ala, Val) | *AE* |
| *Leu or Ileu.(Glu, Ala, Leu, Leu or Ileu) | |
| *Thr.Ala | *X* |
| *Thr.Asp | *Q* |
| Thr.Gly | †*AD* |
| *Thr.Glu | *V* |
| Thr.Pro | †*AA* |
| Thr.(Asp, Arg) | *AQ* |
| *Thr.(Asp, Gly) | *R, U* |
| Thr.(Asp, Val) | †*R* |
| *Thr.(Glu, Ala) | †*Q* |
| *Thr.(Asp, Arg, Arg) | *AU* |
| *Thr.(Asp, Glu, Val) | †*P* |
| Thr.(Asp, Gly, Val) | *R* |
| Thr.(Glu, Gly, Ala) | *AD* |
| Thr.(Asp, Glu, Ala, Val) | *Y* |
| Thr.(Gly, Pro) | *AD* |
| Val.Ala | *AD* |
| Val.Asp | *R* |
| *Val.$CySO_3H$ | *E* |
| Val.Glu | *AA, AB* |
| *Val.(Asp, Glu) | |
| *Val.($CySO_3H$, Ala) | *E* |
| Val.(Glu, Ala) | *Y* |
| Val.(Ileu, Lys) | *AT* |
| Val.($CySO_3H$, Asp, Ala) | *N* |
| Val.(Gly, Thr, Ala, Pro) | *AD* |

also in earlier experiments, using displacement chromatography on ion-exchange resins (Thompson, 1955).

Fractions in the preliminary experiment corresponding exactly to peaks *A* and *B* of the main experiment were examined. Peak *B* consisted practically entirely of free cysteic acid. Peak *A* also contains cysteic acid but in very much smaller quantity than peak *B* and it seems probable that peak *A* is an artifact occurring at the point of emergence of the first hold-up volume of the column.

One of the major components in fraction *AD* gave only glycine on hydrolysis. However, glycine was identified in fraction *M*, which clearly corresponds to its correct position in the elution sequence (cf. Moore & Stein, 1951). It was at first thought that the compound found in fraction *AD* might be Gly.Gly. This was disproved by comparison of the *R* values of the DNP-derivatives on a Celite column buffered at pH 4 and with chloroform as solvent (Perrone, 1951). (*R* of DNP derivative of the glycine compound in fraction *AD*, 0·18; *R* of authentic DNP-glycine, 0·16; *R* of authentic DNP-Gly.Gly, 0·03.) It thus appears that the compound is probably free glycine and the most plausible explanation for its anomalous occurrence in fraction *AD* would seem to be its formation by cleavage of some labile compound in the fraction after the chromatography on ion-exchange resin.

The dipeptide Gly.Lys, detected in experiments using displacement chromatography, was listed in previous communications (Thompson, 1954, 1955). There is now, however, reason to believe that it was, in fact, Lys.Gly. On end-group examination of a peptide containing glycine and lysine in fraction *AQ*, DNP-glycine was again detected in small quantity and it was at first thought that glycine was in the *N*-terminal position, the poor yield of DNP-glycine being attributed to the known lability of this compound to acid hydrolysis (Sanger, 1945). However, on exposing the chromatogram to the vapour of boiling 20% hydrochloric acid (Sanger & Thompson, 1953*a*), a rather elongated spot was revealed under the position occupied by dinitrophenol and another artifact, always present on such chromatograms (run with *tert.*-pentanol–pH 6 buffer) and corresponding to a bis-DNP-lysine control. The dipeptide is therefore Lys.Gly, and the large amount of glycine and virtual absence of lysine or $\epsilon$-DNP-lysine in the aqueous layer confirm this conclusion.

Acher *et al.* (1954) have reported the presence of the peptide Leu.Pro in lysozyme. Since Ileu.Pro was detected in fraction *AH* and there are only two proline residues in this protein, the other being preceded by threonine (cf. Thr.Pro in Table 5), it appears that these workers have failed to distinguish between the two leucine isomers. In the present work clear proof that isoleucine and not leucine was present in this peptide was provided by paper chromatography of a hydrolysate with *tert.*-pentanol–water as solvent system. This conclusion is substantiated by the isolation of the peptide Asp.($CySO_3H$,Pro,Ileu), in which the isoleucine residue was determined in the same way, and by the establishment of the sequence Asp.Ileu.Pro.-$CySO_3H$ (see Table 6).

## DISCUSSION

The results of the experiments described in this paper indicate that elution chromatography on ion-exchange resins has the highest resolving power for the separation of peptides from a partial acid hydrolysate of any method hitherto evaluated. That this procedure alone did not yield pure peptides is a reflexion of the complexity of such a hydrolysate. It may be noted that some 130 peptides have been identified in the lysozyme hydrolysate and the actual number present is probably considerably higher.

These results emphasize that the usual conditions for partial acid hydrolysis produce fairly random splitting of a protein molecule and a peptide mixture of far too great a complexity, so that it is desirable that other methods be worked out for the determination of amino acid sequence of proteins. In the absence of efficient methods for determining sequence by stepwise degradation along a protein chain, more specific methods of cleavage of the chains must be sought to provide a lesser number of fragments for separation and identification. Digestion by proteolytic enzymes to some extent provides such a method, but the cleavage of every susceptible bond is by no means complete (Sanger & Tuppy, 1951; Sanger & Thompson, 1953*b*), with consequent increase in the complexity of the hydrolysate.

Two problems arise in using methods which give rise to longer-chain fragments: The first is the separation of such peptides. It seems likely that elution chromatography on a less highly crosslinked resin (such as '2% crosslinked' Dowex-50) will be successful for this purpose. A preliminary elution chromatogram of a trypsin digest of lysozyme on this resin showed a simpler curve, with a smaller number of well-defined peaks, when compared with the partial acid hydrolysate. It also indicated that successful chromatography of such a peptide mixture can probably be achieved. The second problem is that of determining the structure of these larger peptides, which is far more difficult than with the small peptides from an acid hydrolysate. A preliminary necessity would be a quantitative analysis of each peptide and the isolation of a reasonable quantity of it for the amount of work

Table 6. *Sequences of residues in lysozyme*

1. Thr.Asp.Val.Glu.Ala*
2. Ileu.Glu.Leu.Ala.Leu*
3. Asp.Glu.Ala*
4. Leu.Thr.Ala*
5. Glu.Asp.Ileu*

6. **Thr.Glu.Ala.Gly**
   Thr.(Glu, Ala, Gly)
   Thr. Glu.Ala*

7. **Ser.Asp.Gly.MetO$_2$.Asp**
   Ser.Asp.Gly.MetO$_2$*
   MetO$_2$.Asp

8. **Asp.Ala.MetO$_2$.Lys.CySO$_3$H.Arg**
   MetO$_2$.(Lys, CySO$_3$H, Arg)
   Asp.(Ala, MetO$_2$)
   Ala.(MetO$_2$, Lys)
   MetO$_2$.(Lys, CySO$_3$H)
   Asp. Ala MetO$_2$. Lys
   Ala. MetO$_2$ CySO$_3$H. Arg

9. **Val.Thr.Pro.Gly.Ala**
   Val.(Thr, Pro, Gly, *Ala*)
   Thr.(Pro, Gly)
   Thr. Pro
   (Pro, Gly)

10. **Ser.Asp.Arg**
    Ser.(Asp, Arg)†
    Ser. Asp
    Asp. Arg

11. **Lys.Phe.Glu.Gly**
    Lys.(Phe, Glu)
    (Phe, Glu, Gly)
    Phe. Glu

12. **Arg.CySO$_3$H.Glu.Ala**
    Arg.(CySO$_3$H, Glu)
    CySO$_3$H.(Glu, *Ala*)
    Arg. CySO$_3$H Glu. Ala
    CySO$_3$H. Glu

13. **Ser.Phe.Asp.Glu**
    Ser.(Phe, Asp)
    Phe.(Asp, Glu)
    Phe. Asp

14. **Thr.Asp.Arg.Arg**
    Thr.(Asp, Arg, Arg)
    Thr.(Asp, Arg)†
    Thr. Asp
    Asp. Arg

15. **Thr.Gly.Asp.Val**
    Thr.(Gly, Asp, Val)
    Thr.(Gly, Asp)†
    Thr. Gly

16. **Ser.Val.CySO$_3$H.Ala.Lys.Gly**
    Ser.(Val. CySO$_3$H, Ala, Lys)?
    Val.(CySO$_3$H, Ala)‡
    CySO$_3$H.(Ala, Lys)
    Ala.(Lys, Gly)
    Ser. Val CySO$_3$H. Ala Lys. Gly
    Val. CySO$_3$H Ala. Lys

17. **Gly.CySO$_3$H.Asp**
    Gly.(CySO$_3$H, Asp)†
    CySO$_3$H. Asp

18. **Leu.Gly.Ala.Val**
    Leu.(Gly, Ala, Val)†

19. **Asp.Ileu.Pro.CySO$_3$H**
    Asp.(Ileu, Pro, CySO$_3$H)
    Leu or Ileu.(Pro, CySO$_3$H)§
    Ileu. Pro
    Asp. Ileu

20. **Arg.CySO$_3$H.Lys.Gly**
    Arg.(CySO$_3$H, Lys, Gly)
    Arg.(CySO$_3$H, Lys)
    CySO$_3$H.(Lys, Gly)
    Arg. CySO$_3$H Lys. Gly
    CySO$_3$H. Lys

21. **Ser.Val.Asp.CySO$_3$H.Ala**
    Ser.(Val, Asp, CySO$_3$H)
    Val.(Asp, CySO$_3$H, Ala)
    Ser.(Val, Asp)
    Ser. Val CySO$_3$H. Ala
    Val. Asp

22. **Asp.Leu.CySO$_3$H.Asp**
    Asp.(Leu, CySO$_3$H)
    Leu.(CySO$_3$H, Asp)
    Leu. CySO$_3$H
    Asp. Leu. CySO$_3$H. Asp

23. **Arg.Asp.CySO$_3$H.Ileu**
    Arg.(Asp, CySO$_3$H)
    Asp.(CySO$_3$H, Ileu)
    Arg. Asp. CySO$_3$H. Ileu

24. **Ser.Arg.Leu**
    Ser.(Arg, Leu)
    Ser. Arg
    Arg. Leu

25. **Ser.Asp.CySO$_3$H.Arg.Leu**
    Ser.(Asp, CySO$_3$H, Arg, Leu)
    Asp.(CySO$_3$H, Arg, Leu)
    Asp.(CySO$_3$H, Arg)
    CySO$_3$H.(Arg, Leu)
    Ser. Asp Arg, Leu
    CySO$_3$H. Arg

* Evidence has been presented for these sequences in a previous paper (Thompson, 1955).

† Evidence for these sequences was obtained by partial hydrolysis of the DNP-peptides (see Table 2).

‡ On hydrolysis of the DNP-peptide during end-group examination, a second yellow spot was observed in addition to DNP-valine running a little faster than DNP-cysteic acid in *tert.*-pentanol–pH 6 buffer but readily separated in *n*-butanol–acetic acid. On hydrolysis it yielded DNP-valine and cysteic acid and was thus DNP-Val.CySO$_3$H. This establishes the sequence Val.CySO$_3$H.Ala in the tripeptide.

§ Evidence for this peptide was obtained in the experiments using displacement chromatography (Thompson, 1955) but, as insufficient material was available for confirmation, it was not listed as a definite peptide.

‖ The sequence Ser.Phe.Asp has also been established by Acher, Laurila, Thaureaux & Fromageot (1954).

which structural examination would require. The previous examination of a partial acid hydrolysate does have the advantage of aiding in the interpretation of the results on longer-chain peptides.

The sequences of amino acids in lysozyme deduced from peptides from partial acid hydrolysis identified in this and earlier work (Thompson, 1955) are listed in Table 6, and beneath them the structure of the peptides from which evidence for the sequence is derived. Peptides not used in compiling these sequences suggested a number of additional sequences. They cannot be logically deduced with all other possibilities of combination eliminated but are statistically probable. Further confirmation will be required. They are listed below with the peptides providing evidence for them:

**Ileu.Ser.Leu.Gly**
Ileu.(Ser, Leu, Gly)
Ser.(Leu, Gly)
Ser.Leu

This sequence perhaps adjoins sequence 18 of Table 6 to give

Ileu.Ser.Leu.Gly.Ala.Val

**Asp.Arg.Gly.Leu**
Asp.(Arg, Gly, Leu)
Asp.(Arg, Gly)
Arg.Gly
Asp.Arg Gly.Leu

**$CySO_3H$.Asp.Gly.Arg**
$CySO_3H$.(Asp, Gly, Arg)
$CySO_3H$.(Asp, Gly)
Asp.(Gly, Arg)
$CySO_3H$.Asp
Asp.Gly

This sequence probably adjoins either sequence 17 or 22 of Table 6.

**Ileu.Asp.Ala**
Ileu.(Asp, Ala)
Ileu. Asp
Asp.Ala

**Asp.Ileu.Thr.Ala**
Asp.(Ileu, Thr, Ala)
Ileu.(Thr, Ala)
Asp. Ileu Thr, Ala

**Ser.Ala.Gly.Asp**
Ser.(Ala, Gly, Asp)
Ser.(Ala, Gly)
Ser. Ala

Two other sequences in lysozyme, the *N*-terminal one, Lys.Val.Phe.Gly.Arg (Schroeder, 1952; Acher *et al.* 1954) and Arg.His.Lys (Acher, Thaureaux, Crocker & Fromageot, 1952) have been established.

It is of interest to assess at this stage the extent to which these sequences (1–25, Table 6) contribute to the knowledge of the lysozyme molecule as a whole. It may be observed that the sequences around the two proline and two methionine residues have been established; also three of the three or four phenylalanine residues, the one histidine residue (Acher *et al.* 1952), the ten cysteic acid residues, five of the six lysine residues, and the seven threonine residues. Sequences containing glutamic acid appear to involve seven glutamic acid residues, though the analytical figure for glutamic acid in lysozyme is uncertain (Thompson, 1955).

In addition to the tripeptide and higher sequences deduced above, a number of dipeptides have been isolated but not used in compiling these sequences (1–25, Table 6). Of course they also represent bonds in the lysozyme molecule and are listed below:

| | | |
|---|---|---|
| Ala.Ala | Gly.Leu | Leu.Leu |
| Arg.Asp | Ileu.Arg | Ser.Ala |
| Arg.Gly | Ileu.Asp | Ser.Leu |
| Asp.Asp | Ileu.Val | Val.Ala |

In addition, the dipeptides Tyr.Gly and Gly.Tyr have been reported by Acher *et al.* (1952).

It is of interest to estimate the proportion of the lysozyme molecule which these sequences represent. A very rough assessment may be made by counting the number of bonds allocated in the sequences. The figure is of course high, as condensation of two sequences together may reduce the number of bonds. However, as no alternative method can be readily applied, an approximate estimate of the present position is given in this way. Of the number of bonds in lysozyme, which is of the order of 125–130, 79 have been deduced from the above experiments (see Table 6), a further 6 in the sequences established by other workers, another 14 in the above dipeptides, making a total of 99 bonds allocated. As tryptophan is destroyed during partial acid hydrolysis, one would not expect to locate the 16 bonds involving these residues. The same applies to the 14 bonds involving the amino groups of serine and threonine (9 Ser + 7 Thr − 2 intact Thr bonds), which are preferentially destroyed by acid. Thus about 30 bonds cannot be determined in this hydrolysate. These factors considered, it may be seen that the extent of our knowledge of the lysozyme molecule is approaching completeness in so far as examination of a partial acid hydrolysate can provide such information.

A noticeable blank in the results of the elution experiments is the absence of peptides containing tyrosine [except (Asp, Tyr) in fraction *AH*]. The reason for failure to detect such peptides is not known.

It was previously reported (Thompson, 1954) that the sequence Gly.Phe.Glu.Asp.Ileu occurs in lysozyme. The portion Gly.Phe.Glu was deduced

on the basis of peptides Phe.Glu and (Gly, Phe, Glu), and the peptide Phe.(Asp, Glu) which was thought to belong to the same sequence, leading to the tetrapeptide sequence Gly.Phe.Glu.Asp. The peptide Glu.(Asp, Ileu) extended this to Gly.Phe.-Glu.Asp.Ileu. However, on subsequent isolation of the peptide Lys.(Phe, Glu), and since lysozyme contains probably three phenylalanine residues, it appears that the glycine of (Phe, Glu, Gly) follows the glutamic acid residue, yielding Lys.Phe.Glu.-Gly and the Phe.(Asp, Glu) forms part of the sequence Ser.Phe.Asp.Glu. Evidence is insufficient to indicate whether the next residues are -Asp.Ileu (from Glu.Asp.Ileu, sequence 5 of Table 6) or -Ala (from Asp.Glu.Ala, sequence 3 of Table 6).

Five of the six sequences containing lysine have been established (Table 6). The sixth involves the peptide Val.(Ileu, Lys), but the order of the isoleucine and lysine residues cannot be fixed on available evidence. If the order is Val.Ileu.Lys then the sequence Lys.Phe.Glu.Gly (sequence 11 of Table 6) may follow either this sequence or the sequence Arg.His.Lys established by Acher *et al.* (1952). If the order is Val.Lys.Ileu then it must follow the Arg.His.Lys sequence, giving the extended sequence Arg.His.Lys.Phe.Glu.Gly.

On inspection of the sequences thus established, no regularity in the structure of this protein has become apparent. The arrangement of amino acids follows very much a random distribution (except that the recurrence of the dipeptide sequence Glu.Ala on four occasions would appear to be more than chance distribution would yield).

Although the full structure of the molecule is not known, it seems unlikely that amino acid sequence determination alone will provide the clue as to the requirements of the enzyme for its activity. Such an understanding will only be reached when further studies, testing the effect of modification of the molecule on its biological activity, have been carried out. As a basis for this, knowledge of the sequence of amino acids in the molecule is of prime importance.

## SUMMARY

1. A partial acid hydrolysate of egg-white lysozyme has been fractionated by gradient elution chromatography on sulphonated-polystyrene resin with ammonium formate and acetate buffers.

2. A large number of peptides have been isolated by chromatography of these fractions on paper, and their structures determined.

3. A number of sequences in the lysozyme molecule have been deduced from these results.

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# A Search for Specific Chemical Methods for Fission of Peptide Bonds

## 2. EXPERIMENTS INVOLVING REDUCTION OF *C*-TERMINAL RESIDUES*

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In the first paper of this series (Elliott, 1952) it was shown that *N*, *O*-acyl migration could be used to bring about cleavage of the silk fibroin molecule at some of the serine residues, but that other peptide bonds involving serine remained resistant to the reagent. It seemed possible that this resistance may have been due to steric hindrance by bulky side chains in the neighbourhood of serine residues. In view of the very high content of glycine and alanine in silk fibroin it could be assumed that they are linked frequently to serine and that it is these bonds which undergo the acyl migration reaction, whereas bonds involving serine and amino acids with larger side chains remained unaffected. In order to test this hypothesis a quantitative *C*-terminal end-group method of wide scope was needed. No such method existed when this work commenced, but reduction of the *C*-terminal residues with a metallo-hydride seemed promising. In addition it was hoped that reduction of the carboxyl groups of peptides would form the starting point for a stepwise degradation method as outlined below.

Fromageot, Jutisz, Meyer & Pénasse (1950) had already shown that lithium aluminium hydride reduced the free carboxyl groups of proteins to primary alcohol groups and that amino alcohols were obtained on subsequent hydrolysis, but the results obtained on insulin were far from quantitative. Chibnall & Rees (1951, 1952) had used lithium borohydride to determine the distribution of amide groups in insulin. Our initial experiments consisted in the reduction of a few simple peptide derivatives by lithium aluminium hydride and lithium borohydride, in connexion with the stepwise degradation method. Attempts were then made to develop a reliable end-group method for large peptides and proteins.

## RESULTS

### *Reduction of simple peptides and related compounds by mixed metallo-hydrides*

In some of these experiments benzoylated glycyl peptides were used; these were readily prepared by the condensation of 2-phenyloxazolone with amino acids in the presence of aqueous alkali, and were converted into their methyl esters by the action of diazomethane. At first, attempts were made to use lithium aluminium hydride for reduction on account of its comparatively low cost. In most cases no attempt was made to isolate the main products as crystalline substances, but instead the crude material was hydrolysed and submitted to paper chromatography. It was hoped that all products would be detected by this method and not merely those present in largest amount. Reduction at the boiling point of the solvent, tetrahydrofuran (THF), even for a short while, was not satisfactory. Under these conditions and after acid hydrolysis, benzoylglycylalanine methyl ester yielded a considerable amount of *N*-benzylglycine in addition to glycine and alaninol. There was also present a fourth ninhydrin-positive substance which may have been a secondary amine derived from the reduction of the glycylalanine linkage. When methyl hippurate was treated with lithium aluminium hydride in boiling THF 2-benzylaminoethanol was produced in 65% yield. The spot due to *N*-benzylglycine persisted in all chromatograms obtained from reductions of benzoylglycyl peptide esters carried out at room temperature or at 0° for several hours. Benzoylglycylalanine methyl ester was not reduced by lithium aluminium hydride at −50°. Since this work was completed, Jutisz, Meyer & Pénasse (1954) have carried out similar work with lithium aluminium hydride and hippuric acid or its methyl ester. They observed both reduction of the benzamido group to a benzylamino group and reductive cleavage of the C—N linkage of the amide group. Their experiments also showed that 60–90% reduction of the end groups of dipeptides resulted when they were treated for 2 hr. at 0° with lithium aluminium hydride, but traces of the second amino acid appeared as an amino alcohol even in these reductions. In view of these results it seems likely that some peptide bonds in proteins would be attacked by lithium aluminium hydride.

Reductions with lithium borohydride gave much more satisfactory results. The end group of benzoylglycylalanine methyl ester was reduced completely with no side reactions when treated with

* Part 1: Elliott, D. F. (1952).

lithium borohydride for 18 hr. at 0°. It was also found that *N*-benzoylethanolamine and *N*-benzoylglycylethanolamine were unaffected by the reagent under the same conditions. When reduced at the boiling point of THF by lithium borohydride, however, benzoylglycylalanine methyl ester gave rise to a new spot on the chromatogram which had an $R_F$ value identical with that of ethanolamine. No attempt was made to identify this substance, but experiments carried out later leave little doubt that it was ethanolamine; reductive cleavage of the peptide bond was not envisaged at that time. These preliminary experiments indicated that lithium borohydride was a satisfactory reducing agent for simple peptides, but that further study of the side reactions occurring under vigorous conditions might be necessary. This problem is discussed below. Recently, Grassmann, Hörmann & Endres (1953) have described the reduction of peptides by lithium borohydride at the boiling point of THF.

*Attempts to devise a stepwise degradation of peptides*

A number of attempts were made to develop a stepwise degradation based on the acyl migration reaction used in the degradation of silk fibroin (Elliott, 1952). The following scheme of reactions was envisaged:

$$\ldots NH.CHR'.CO.NH.CHR.CO_2H \xrightarrow[\text{and reduction}]{\text{esterification}}$$

$$\ldots NH.CHR'.CO.NH.CHR.CH_2.OH \xrightarrow{\text{acyl migration}}$$

$$\ldots NH.CHR'.CO.O.CH_2.CHR.NH_2 \xrightarrow{\text{acylation}}$$

$$\ldots NH.CHR'.CO.O.CH_2.CHR.NH.CO.R'' \xrightarrow[\text{hydrolysis}]{\text{alkaline}}$$

$$\ldots NH.CHR'.CO_2H + HO.CH_2.CHR.NH.CO.R''$$

This would have constituted a valuable degradative procedure for the peptides containing *N*-terminal serine or threonine resulting from the fission of proteins by the acyl migration method. A great deal of time was devoted to studying the use of hydrogen chloride in anhydrous methanol and nitromethane, which could be readily removed at the end of the reaction, to bring about the *N*, *O* rearrangement. Although many different conditions of time and temperature and various concentrations of hydrogen chloride in methanol were examined, the rearrangement was never quantitative and was generally accompanied by side reactions. It was also found that free ethanolamine was produced by the action of methanolic hydrogen chloride on *N*-benzoylglycylethanolamine; this probably occurred by alcoholysis of the *O*-peptidyl linkage. Attempts to force the latter reaction to completion by using more vigorous conditions resulted in partial cleavage of the benzoyl group. These experiments are summarized in Table 1. Desnuelle & Bonjour (1951) showed that a saturated solution of hydrogen chloride in methanol caused cleavage of peptide bonds in addition to those involving serine and threonine in proteins when the reaction time was prolonged.

It was found that after 20 hr. at room temperature in nitromethane–hydrogen chloride the theoretical quantity of amino N was formed from *N*-benzoylglycylethanolamine. The reagent itself was unstable and gave rise to a crystalline ppt. after about 3 days. It was shown, however, that after 20 hr. there was insufficient of this substance present to affect the amino N determinations. Paper chromatography revealed, however, that an appreciable quantity of free ethanolamine was present in the product. The mechanism by which this substance is produced under anhydrous conditions is not clear.

The product obtained by lithium borohydride reduction in the cold of benzoylglycylalanine methyl ester gave similar results to the ethanolamine derivative on rearrangement by the two methods described above.

Cold concentrated sulphuric acid, which was found to be a satisfactory reagent in the protein

Table 1. *Products obtained (indicated by ×) on treatment of N-benzoylglycylethanolamine with methanolic hydrogen chloride under various conditions*

Additional, unidentified substances were obtained in experiments carried out at 100°.

| Normality of HCl soln. used | Mol. prop. HCl used | Temp. of reaction (°) | Time (min.) | Ethanolamine | Glycine methyl ester | *O*-Benzoylglycylethanolamine |
|---|---|---|---|---|---|---|
| 3 | 5 | 100 | 5 | × | × | × |
| 1 | 5 | 100 | 20 | × | × | × |
| 0·3 | 5 | 100 | 90 | × | × | × |
| 3 | 1 | 100 | 10 | × | Trace | × |
| 3 | 1 | 100 | 90 | × | × | × |
| 1 | 1 | 100 | 20 | × | × | × |
| 3 | 1 | 20 | 1020 | × | — | × |
| 3 | 5 | 20 | 1020 | × | — | × |
| 1 | 5 | 20 | 1020 | × | — | × |

field, was unsuitable for small peptides, which do not lend themselves to straightforward isolation from this reagent. Orthophosphoric acid was unsatisfactory for the same reason and glacial acetic acid at 100° did not cause rearrangement. Fuming sulphuric acid in nitromethane caused no production of amino N from benzoylglycylethanolamine.

The formation of free amino alcohols during rearrangement gave rise to difficulties in the acetylation stage. When acetylation of the rearranged peptide was carried out at pH 5 according to the procedure described by Elliott (1952) the amino group of the *O*-peptidyl form was blocked, but that of the free amino alcohol was unattacked. Since the quantity of amino alcohol present was generally about 20 % of the total end group, it did not seem justifiable to ignore this amount. It would then have been necessary either to carry out a second acetylation at a higher pH or to devise a technique for the isolation of the amino alcohol. In either event the process would have become rather involved and unsuitable for small-scale operation. This work was therefore discontinued.

*Isolation of amino alcohols from mixtures with amino acids and inorganic salts*

The isolation of pure amino alcohols from the mixtures obtained after metallo-hydride reduction and hydrolysis of peptides and proteins is not easy, partly on account of the presence of inorganic salts formed from the metallo-hydrides. In many cases electrolytic desalting was necessary before isolation procedures could be carried out (Chibnall & Rees, 1953; Fromageot & Jutisz, 1953). There appears to be no solvent, except ether, in which amino acids and inorganic residues from the metallo-hydrides are insoluble and the amino alcohols soluble; even in this solvent, ethanolamine and the aminodiols are practically insoluble. These amino alcohols were more easily extracted by repeated shaking of a hydrolysate, made alkaline by potassium carbonate, with 1 % ethereal anisaldehyde. This was presumably due to the formation of Schiff bases which were more soluble in the organic solvent. The amino alcohols were recovered in the pure state from the ethereal extracts by evaporation and steam distillation of the acidified residue to remove the anisaldehyde.

Some attempts were then made to develop chromatographic procedures for the separation and identification of amino alcohols. Dr A. T. James of this Institute kindly carried out gas–liquid chromatography, but found that, whereas the simpler amino alcohols were readily separated from each other, the aminodiols gave rise to trailing peaks in all the systems used. Liquid–liquid chromatography, using 'Hyflo Super-Cel' as support, aqueous potassium hydroxide as the stationary phase and various organic solvents as the moving phase was also investigated but the degree of resolution was rather poor.

The conversion of the amino alcohols into their 2:4-dinitrophenyl (DNP) derivatives was next investigated (cf. James & Synge, 1951; Waldkötter, 1938); it seemed likely that these substances would be readily soluble in ether and therefore easily isolated from hydrolysates. They were formed by means of 1-fluoro-2:4-dinitrobenzene under various conditions, but it was found that serinol gave rise to a small amount of a yellow substance in addition to the *N*-substituted derivative. This was probably the *ON*-bis-dinitrophenyl or the *OO'N*-trisdinitrophenyl derivative. The reaction of the hydroxyl group of ethanol with fluorodinitrobenzene has been observed by Zahn & Wurtz (1952). The formation of a by-product in the preparation of DNP-amino alcohols was very undesirable; the yellow compound from serinol, for instance, had the same *R* value as *N*-DNP-threoninol in one of the solvent systems used for chromatography.

Eisen, Belman & Carsten (1953) have shown that sodium 2:4-dinitrobenzenesulphonate slowly reacts with protein amino groups at pH 10–11 to give DNP derivatives. This reaction, which occurs by elimination of a molecule of sodium sulphite, is analogous to the formation of amino-substituted quinolines by heating 2- and 4-quinoline sulphonic acids with bases (Walker, 1947). It seemed possible that sodium 2:4-dinitrobenzenesulphonate (DNBS) would react only with the amino groups of amino alcohols. An apparent disadvantage of the reagent was the high pH needed for the reaction, but it was soon evident that DNP-amino alcohols have a higher stability to alkali than DNP-amino acids. A number of different reaction conditions were examined with alaninol as test substance, a quantitative yield of the DNP derivative being obtained when it was heated at 95° for 20 hr. with DNBS in the presence of borax solution. These conditions were also suitable for the isolation of amino alcohols from hydrolysates; serinol, ethanolamine and alaninol were recovered almost quantitatively in this manner after being mixed with 40 parts of silk-fibroin hydrolysate. There appeared to be no substitution of the hydroxyl groups under these conditions; even tyrosinol gave only the *N*-substituted derivative.

*Chromatography of DNP-amino alcohols.* Since this work was commenced the isolation and chromatography of DNP-amino alcohols has been described by several workers (Grassmann, Hörmann & Endres, 1954; Jatzkewitz & Tam, 1954; Luck & Wilcox, 1953; Jutisz, Privat de Garilhe, Suquet & Fromageot, 1954). The choice of solvents for chromatography of DNP-amino alcohols is rather limited on account of their high solubility in

organic solvents and low solubility in water. This was overcome by Jutisz, Privat de Garilhe, Suquet & Fromageot (1954) by the use of reversed-phase chromatography and by Grassman, Hörmann & Endres (1954) and Luck & Wilcox (1953), who used adsorption columns of silicic acid. The use of ethylene glycol–benzene on columns of silica gel for separation of certain DNP-amino acids was described by Sanger (1945). DNP-amino alcohols were found to be easily soluble in ethylene glycol and related polyhydroxy compounds; when these were used as stationary phases a wide choice of solvent systems for column partition chromatography was made possible. Using Hyflo Super-Cel as a support, the columns were easy to pack, only slightly affected by temperature changes, and could be used many times without loss of resolving power. After elution from the column the bands were estimated spectrophotometrically at 350 mμ. Details of the column procedure and the solvent systems used are given in Tables 2 and 3.

*Lithium borohydride as an end-group reagent*

The first objective was the determination of the *C*-terminal residues of the peptides obtained from silk fibroin by fission at serine residues by the following series of reactions:

$$\text{Silk fibroin} \xrightarrow[\text{dialysis}]{H_2SO_4,\ CH_3.CO.O.CHO} \text{modified protein,}$$

$$\xrightarrow[\text{dialysis}]{\text{alkaline hydrolysis}} \text{diffusible formyl peptide mixture.}$$

It was hoped that this would lead to a better understanding of the acyl migration reaction and at the same time perhaps some light would be shed on the structure of silk fibroin. In this work the mixture of peptides used was prepared as described by Elliott (1952), except that formic acetic anhydride ($H_3C.CO.O.CHO$) was used in place of acetic anhydride. In order to find out how many *C*-terminal residues such a mixture contained, a

Table 2. R *values of DNP-amino alcohols in various solvent systems (see Table* 3)

Columns were 12 mm. in diam. and contained 6 g. acid-washed Hyflo Super-Cel mixed with 3 g. stationary phase, except for system no. 6 in which 4 g. stationary phase were used. Chloroform used in system no. 7 was washed with water to remove ethanol and used saturated with water. Stationary phases were ethylene glycol, glycerol, trimethylene glycol or tetramethylene glycol.

| | Solvent system no. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *N*-DNP Derivative of | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Serinol | 0·24 | 0·11 | 0·02 | — | — | — | 0·25 | 0·15 | — |
| Aspartidiol | — | 0·14 | — | — | — | — | 0·58 | 0·18 | 0·14 |
| Glutamidiol | 0·20 | 0·13 | — | — | — | — | 0·64 | 0·14 | 0·11 |
| Threoninol | 0·56 | 0·23 | — | — | — | — | 0·72 | 0·25 | — |
| Tyrosinol | 0·70 | 0·25 | — | — | — | — | 0·58 | — | — |
| Lysinol (mono-) | — | — | 0·21 | — | — | — | — | — | — |
| Ethanolamine | — | 0·88 | 0·50 | — | — | — | — | — | — |
| Alaninol | — | — | 0·70 | — | — | 0·37 | — | — | — |
| Ammonia | — | — | 0·70 | — | — | 0·27 | — | — | — |
| Lysinol (bis-) | — | — | 0·90 | — | — | — | — | — | — |
| Prolinol | >1·20 | >1·20 | 1·04 | 0·10 | 0·35 | — | — | — | — |
| Valinol | — | — | 1·05 | 0·33 | 0·55 | — | — | — | — |
| Leucinol | — | — | 1·20 | 0·60 | 0·78 | — | — | — | — |
| Isoleucinol | — | — | — | 0·60 | 0·80 | — | — | — | — |
| Phenylalaninol | — | — | 1·10 | 0·23 | 0·55 | 0·87 | — | — | — |
| *O*-Methyltyrosinol | — | — | — | 0·20 | 0·52 | 1·00 | — | — | — |

Table 3. *Composition of solvent systems (parts by volume)*

| | Solvent system no. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Ethylene glycol | 2 | 2 | Excess | 2 | 2 | 2 | — | — | — |
| Benzene | 20 | 20 | Excess | — | 20 | — | — | 20 | 20 |
| *cyclo*Hexane | — | — | — | 20 | 20 | — | — | — | — |
| Acetone | 2 | 1 | — | 1 | — | — | — | 1 | 1 |
| Chloroform | — | — | — | — | — | 12 | Excess | — | — |
| Carbon tetrachloride | — | — | — | — | — | 12 | — | — | — |
| Glycerol | — | — | — | — | — | — | Excess | — | — |
| Trimethylene glycol | — | — | — | — | — | — | — | 2 | — |
| Tetramethylene glycol | — | — | — | — | — | — | — | — | 2 |

small portion was treated with 15% (w/v) methanolic hydrogen chloride for 30 min. at room temperature, when the formyl groups were removed without peptide bond fission (Elliott, 1953). The *N*-terminal residues were estimated by amino N determinations and the *N*-terminal serine residues by the periodate technique of Rees (1946). The figure obtained for the total *N*-terminal residues represented also the maximum possible value for the *C*-terminal residues, that for *N*-terminal serine being the minimum value.

Table 4. *Yields of amino alcohols produced by* $LiBH_4$ *reductions under various conditions of the formyl peptide mixture obtained by degradation of silk fibroin*

Results expressed in terms of m-equiv./g. of peptide.

| Pretreatment of peptide mixture | Time (hr.) | Temp. (°) | Yield of amino alcohol | | | |
|---|---|---|---|---|---|---|
| | | | Ethanolamine | Alaninol | Serinol | Total |
| Esterification | 72 | 20 | 0·32 | 0·09 | 0·02 | 0·43 |
| Esterification | 12 | 70–75 | 0·50 | 0·215 | 0·025 | 0·74 |
| Esterification | 18 | 70–75 | 0·77 | 0·40 | 0·065 | 1·235 |
| None | 18 | 70–75 | 0·195 | 0·16 | 0·04 | 0·395 |

Total *N*-terminal residues, 0·93; *N*-terminal serine residues, 0·84.

The formylpeptides were esterified by diazomethane before reduction. At first, attempts were made to bring about reduction of the esterified peptide mixture with lithium borohydride in THF at room temperature, but even after 3 days only about 50% reduction had occurred. It was clear that these poor results were due to the insolubility of the peptide mixture in the solvent and the effect of raising the temperature was therefore examined. When the reaction was carried out at 70–75° for 12 hr., the yield of amino alcohols approached the theoretical quantity, but in an 18 hr. reduction the yield was far greater than theoretical. When a similar reduction was carried out on the peptide mixture without previous diazomethane treatment the same amino alcohols (ethanolamine, alaninol and serinol) were obtained in about one-third of the quantity obtained from the esterified peptides. That this result was not entirely due to reduction of the free carboxyl groups was demonstrated by a similar treatment of hippuric acid, whereby only a 12% yield of ethanolamine was obtained. These results are summarized in Table 4, where the values obtained for the total *N*-terminal residues and the *N*-terminal serine residues are also given.

It was then found that lysozyme, which had been recrystallized four times, gave rise to ethanolamine and alaninol as well as several other amino alcohols, when treated with lithium borohydride in THF at 70–75° (see Table 5). There is no doubt that, with the possible exception of leucinol, these arose by fission of the peptide chain. It has been shown by two independent methods that the *C*-terminal residue of lysozyme is leucine (Thompson, 1952; Harris, 1952; Ohno, 1953). When a portion of the lysozyme, after reduction by lithium borohydride, was treated with fluorodinitrobenzene under the usual conditions of Sanger (1945) and the product hydrolysed, a number of different DNP-amino acids were produced. Thus there appeared to be no particular specificity in this reductive breakdown.

Some attempts were made to devise conditions whereby lithium borohydride could be used in aqueous media in which the protein would be soluble. The reagent decomposed slowly when added to water, but it was found possible to bring about partial reduction of methyl hippurate in aqueous methanolic solution by addition of an excess of reagent in several portions over a long period of time. The effects of various catalysts were also investigated. In none of these experiments could the yield of ethanolamine be raised above about 40%. Unfortunately no DNP-leucinol was produced from esterified lysozyme after reduction in aqueous solution.

Table 5. *Amino alcohols obtained from lysozyme on treatment with* $LiBH_4$ *in THF at 70–75° for* 18 hr.

Results expressed as moles/mole of enzyme (14700 g.)

| Amino alcohol | Yield |
|---|---|
| Ethanolamine | 1·23 |
| Alaninol | 2·02 |
| Serinol | 0·48 |
| Leucinol or isoleucinol | 0·74 |
| Valinol | 0·25 |
| Phenylalaninol | 0·17 |

## EXPERIMENTAL

### *Synthesis of model compounds*

Melting points are uncorrected.

*N-Benzoylethanolamine.* Prepared by the method of Knorr & Rössler (1903).

*N-Benzoylglycylethanolamine.* 2-Phenyloxazolone (5·3 g.) was added in small portions during 10 min. to ethanolamine (2 g.), dissolved in a mixture of water (10 ml.) and ethanol (20 ml.), with vigorous shaking. A clear soln. was obtained

and after it had been kept for 1·5 hr. at room temp. it was evaporated to dryness under reduced pressure. The residual oil of *N-benzoylglycylethanolamine* was crystallized from a mixture of ethanol and benzene and had m.p. 126°. (Found: C, 59·6; H, 6·2; N, 12·6. $C_{11}H_{14}O_3N_2$ requires *C*, 59·5; H, 6·3; N, 12·6%.) The yield was 4·45 g. (61%).

DL-*Benzoylglycylalanine.* 2-Phenyloxazolone (1·6 g.) was added in small portions during 10 min. to a vigorously shaken soln. of DL-alanine (0·89 g.) in N-NaOH (10 ml.) and ethanol (10 ml.). Ethanol was removed by evaporation at reduced pressure and the soln. acidified with conc. HCl. After the soln. had stood for 45 min. the ppt. was filtered off, washed with water and crystallized from boiling water. The yield was 1·97 g. (79%) and the m.p. 200° (Curtius & Lambotte (1904), report 202° for this compound). (Found: N, 10·9. Calc. for $C_{12}H_{14}O_4N_2$: N, 11·2%.)

DL-*Benzoylglycylphenylalanine.* This was prepared in a similar manner to the alanine derivative in 43% yield and had m.p. 202° after crystallization from hot water (Curtius & Müller (1904), report m.p. 172° for this compound). (Found: N, 8·7. Calc. for $C_{18}H_{18}O_4N_2$: N, 8·6%.)

L-*Benzoylglycylproline.* 2-Phenyloxazolone (0·8 g.) was added in small portions during 5 min. to a vigorously shaken soln. of L-proline (0·57 g.) in N-NaOH (5 ml.) and ethanol (5 ml.). The clear soln. was allowed to stand for 1 day. The ethanol was then removed under reduced pressure and the aqueous soln. acidified with cold conc. HCl (Congo red). No ppt. formed. The soln. was evaporated to dryness under reduced pressure and the residue extracted with ethanol. L-*Benzoylglycylproline* (0·53 g., 39%), m.p. 205°, separated from the ethanolic extract on addition of ether. It was recrystallized from ethanol and had m.p. 207°, $[\alpha]_D^{20} + 8{\cdot}75 \pm 0{\cdot}5°$ in 0·042 N-NaOH (*c*, 0·575). (Found: C, 60·4; H, 6·0; N, 9·6. $C_{14}H_{16}O_4N_2$ requires C, 60·9; H, 5·8; N, 10·2%.)

*N-Benzylglycine.* This was synthesized by the method of Baker, Ollis & Poole (1949).

### *Reduction of peptides and their esters by* $LiAlH_4$ *and* $LiBH_4$

*Purification of THF.* The solvent was allowed to stand 24 hr. over NaOH flakes and was then distilled over fresh NaOH flakes. The distillate was refluxed 6 hr. with Na wire and distilled a second time. Last traces of water were removed by refluxing 1 hr. with a small quantity of $LiAlH_4$ and distilling into a carefully dried receiver. It was stored in the dark over Na wire to prevent formation of peroxides.

*Preparation of solutions of* $LiAlH_4$. The reagent was obtained in the form of solid masses of irregular size from which a portion (2·5 g.) was broken, rapidly ground and added to THF (50 ml.) in a flask closed with a $CaCl_2$ tube. After refluxing for 1 hr. the soln. was rapidly filtered through dry kieselguhr and stored in a stoppered bottle kept in a sealed box containing $CaCl_2$ as a desiccant. The $LiAlH_4$ content of the soln. was roughly estimated by the vol. of $H_2$ evolved on decomposition by 10% aqueous dioxan in a Warburg flask connected to a gas burette. In general the $LiAlH_4$ content of the solid reagent was about 80%. A soln. prepared in this way was therefore approximately molar.

*Chromatography of the reduction products.* At the end of a reduction the mixture was cooled in ice and unchanged reagent decomposed by addition of a small excess of water. The soln. was refluxed 10 min., cooled again and the ppt. removed by centrifuging. The soln. was evaporated to dryness and a portion hydrolysed with conc. HCl at 100° for 18 hr. The hydrolysate was submitted to paper chromatography, using the butanol–acetic acid–water mixture of Fromageot *et al.* (1950) and the sheet sprayed with ninhydrin. A hydrolysate of the starting material was used as a control.

Esterification of the peptides was carried out by addition of excess of ethereal diazomethane to a suspension in a 1:1 (by vol.) mixture of ether and methanol. When effervescence had ceased, the excess of reagent was decomposed with a few drops of acetic acid and the solvent evaporated.

*Reduction of* DL-*benzoylglycylalanine methyl ester by* $LiAlH_4$. (*a*) *At the b.p. of THF.* The ester (0·88 g.) dissolved in THF (5 ml.) was added dropwise during 20 min. to a boiling M soln. of $LiAlH_4$ in THF (10 ml.). The soln. turned a deep orange colour. After heating for 1·5 hr. a portion of the product was hydrolysed and examined chromatographically. Glycine ($R_F$ 0·05), alaninol ($R_F$ 0·24) and *N*-benzylglycine ($R_F$ 0·40) were present in addition to an unidentified substance, $R_F$ 0·58. Another portion of the reduction product was hydrolysed with 5 N-$H_2SO_4$ at 100° for 18 hr. The liberated benzoic acid was extracted with ether and estimated by titration. Only about 20% of the theoretical amount of benzoic acid was present.

(*b*) *At* 0°. Reduction was carried out as above, except that 5 mol. prop. of the reagent were used and the reaction mixture was cooled in ice. After remaining at 0° for 18 hr. the product was examined as before. *N*-Benzylglycine was present in addition to glycine and alaninol and was still present in the product when the reaction time was decreased to 2 hr.

(*c*) *At* −50°. As in (*a*) except that the $LiAlH_4$ soln. was cooled to −50° during the addition of the ester and the reaction mixture then maintained for 4 hr. at −50°. No alaninol could be detected in the hydrolysed product.

*N*-Benzylglycine was also obtained when benzoylglycylalanine was reduced by $LiAlH_4$ at room temp. for 18 hr.

*Reduction of methyl hippurate at the b.p. of THF.* The ester was reduced exactly as for benzoylglycylalanine methyl ester and the product treated with ethereal picric acid. *N*-Benzylaminoethanol picrate was produced in 65% yield and had m.p. 136° (Gabriel & Stelzner (1896), recorded m.p. 135–136° for this compound). (Found: C, 48·0; H, 4·5; N, 14·7. Calc. for $C_{15}H_{16}O_8N_4$: C, 47·3; H, 4·2; N, 14·7%.)

When DL-benzoylglycylphenylalanine methyl ester was reduced by $LiAlH_4$ at 0° for 3 hr. *N*-benzylglycine was found in the hydrolysate of the product, but was absent from a similar reduction of L-benzoylglycylproline.

*Reduction of* DL-*benzoylglycylalanine methyl ester by* $LiBH_4$. The sample of $LiBH_4$ used in these experiments was almost pure and it was assumed that the wt. of $LiBH_4$ in soln. was equal to the amount weighed out. It dissolved rather slowly in THF at room temp. and left a small amount of white residue.

(*a*) *At* 0°. A solution of 1 m-mole of the ester in 5 ml. THF was cooled to −50° and to it was added a solution of $LiBH_4$ (5 mol. prop., 110 mg.) in THF (15 ml.) also cooled to −50°. The mixture was then maintained at 0° for 18 hr. At the end of this time the soln. was cooled to −50° and water (0·1 ml.) added. The soln. was then refluxed 2 min., cooled and filtered. The filtrate was evaporated to dryness

and hydrolysed in the usual way. A chromatogram showed only glycine and alaninol. It was also shown that *N*-benzoylethanolamine and *N*-benzoylglycylethanolamine were unaffected by the reagent under the same conditions.

(*b*) *At the b.p. of THF.* The ester and reagent were mixed as in (*a*) and then refluxed 4 hr. The chromatogram showed the presence of glycine and alaninol and in addition a substance, $R_F$ 0·15. Ethanolamine also had $R_F$ 0·15 in the same solvent mixture.

### *Acyl migration experiments on reduced peptides*

Amino N was estimated in the volumetric apparatus of Van Slyke (1911). After addition of the reagents and the sample to the reaction vessel the mixture was shaken for 1 min. at room temp. (18–22°) and reaction allowed to proceed for 1 hr. without shaking. After further shaking for 1 min. the volume of nitrogen evolved was measured in the usual way. This procedure gave quantitative results with *cyclo*hexylamine, alaninol, ethanolamine and *O*-benzoylethanolamine hydrochloride.

The presence of the *O*-peptidyl form of a reduced peptide was demonstrated by application of duplicate samples from a rearrangement to a sheet of chromatography paper and exposing one sample to $NH_3$ vapour for a few seconds. The sheet was then developed in the solvent of Fromageot *et al.* (1950) and sprayed with ninhydrin. The spot due to the rearranged peptide was absent in the sample treated with ammonia, whereas amines formed by side reactions were unaffected. Glycine methyl ester gave a yellow spot with ninhydrin.

The experiments with *N*-benzoylglycylethanolamine, recorded in Table 1, were carried out on 50 mg. of the substance in sealed glass tubes. At the end of the reaction the tubes were opened and the methanol and HCl removed in a vacuum desiccator over NaOH and $H_2SO_4$. In the experiments carried out at 100° minor amounts of ninhydrin-reactive substances were present in addition to those recorded; these were not identified. An amino N determination on the product obtained by treating *N*-benzoylglycylethanolamine with 30 mol. prop. of methanolic HCl showed that only 60% of the theoretical amount was present. Rearrangements with HCl in nitromethane were carried out by suspending the substance in the dry solvent and passing in dry HCl until the soln. was saturated. The clear soln. which was obtained was allowed to stand for at least 20 hr. at room temp. The product was isolated in the same manner as described for rearrangements in methanol–HCl.

### *Synthesis of DNP-amino alcohols*

*Amino alcohols.* DL-Alaninol, DL-valinol, DL-leucinol, DL-isoleucinol, DL-phenylalaninol and DL-glutamidiol were prepared from the amino acid esters by the method of Karrer, Portmann & Suter (1948) and were purified as their oxalates, with the exception of glutamidiol which was used as a crude oil. The serinol oxalate used was a gift from Dr H. R. V. Arnstein. DL-Tyrosinol hydrochloride and its *O*-methyl derivative were synthesized by the method of Berlinguet (1954).

DL-Threoninol was prepared as follows. DL-Benzoylthreonine was esterified with ethereal diazomethane and the ester crystallized from ether. *DL-N-Benzoylthreonine methyl ester* had m.p. 85–86°. (Found: C, 61·3; H, 6·4. $C_{12}H_{15}O_4N$ requires C, 60·8; H, 6·4%.) The ester (1 g.) was added to a soln. of $LiBH_4$ (0·2 g.) in THF (20 ml.). The mixture effervesced slightly and became warm. It was allowed to stand 2 days and the excess reagent decomposed by addition of water and a few drops of conc. HCl. The solvent was removed under reduced pressure and the residue hydrolysed with 6N-HCl (10 ml.) at 105° for 16 hr. The hydrolysate was cooled, filtered from benzoic acid and the filtrate evaporated to dryness. The crude hydrochloride was used for the preparation of the DNP derivative as described later.

DL-Dibenzoyllysine, prepared by the method of Fischer & Weigert (1902), was dissolved in 50% (v/v) aqueous methanol and treated with excess of diazomethane in ether. The crude ester (0·01 mole) was reduced by refluxing 4 hr. with $LiBH_4$ (100 mg.) in THF (20 ml.). The crude DL-lysinol hydrochloride was obtained as in the case of threonine.

DL-Benzoylaspartic acid (Fischer, 1899) was esterified with ethereal diazomethane and the product crystallized from ether. *DL-Benzoylaspartic acid dimethyl ester* had m.p. 83·5°. (Found: N, 5·65. $C_{13}H_{15}O_5N$ requires N, 5·3%.) The ester was reduced as in the case of DL-threoninol and the hydrochloride of DL-aspartidiol isolated in the usual way.

*Preparation of DNBS* (cf. Fierz, 1920). 1-Chloro-2:4-dinitrobenzene (20·2 g.), powdered $Na_2SO_3,7H_2O$ (25 g.), ethanol (65 ml.) and water (15 ml.) were mixed and stirred under reflux on the steam bath for about 8 hr. The orange solution was filtered hot and cooled at 0° for several hr. The product was filtered, washed with an ethanol–water mixture (13:3, by vol.) and purified by crystallization from water, using 2 ml. per g. of solid. The yield was 10 g.

*Reaction of DNBS with amino alcohols.* For preparative purposes it was convenient to use aq. $Na_2CO_3$ as the reaction medium. A short reaction period sufficed to give yields of 80–85%.

The amino alcohol (free base, hydrochloride, or oxalate, 0·5 m-mole) was mixed with DNBS (300 mg., 100% excess), N-$Na_2CO_3$ (2·5 ml.) and water (2·5 ml.). The mixture was heated in a sealed tube at 100° for 1 hr. with occasional shaking until the solids had dissolved. The DNP derivative frequently separated as an oil from the soln. during the reaction. At the end of the reaction, the mixture was diluted with an equal volume of water and the DNP-amino alcohol extracted with chloroform and then crystallized from the appropriate solvent. The following products were obtained (solvent for crystallization in parenthesis):

*N*-DNP-Ethanolamine (benzene), m.p. 89°. It gave no depression in m.p. with a specimen prepared in a different manner by Dr A. T. James.

*N*-DNP-DL-Alaninol (benzene–light petroleum, b.p. 60–80°), m.p. 94–95°. (Found: N, 17·2. Calc. for $C_9H_{11}O_5N_3$: N, 17·4%.) (Cf. Grassmann *et al.* 1953; Luck & Wilcox, 1953; Jatzkewitz & Tam, 1954.)

*N*-DNP-DL-Valinol was an oil which slowly solidified and had m.p. 67–71° in the crude state. It could not be recrystallized but behaved as a single substance in solvent systems nos. 3, 4 and 5 (Table 2). (Cf. Grassmann *et al.* 1953; Jatzkewitz & Tam, 1954.)

*N*-DNP-DL-Isoleucinol was an oil, but behaved as a single substance in solvent systems nos. 4 and 5.

*N*-DNP-DL-Leucinol (light petroleum), m.p. 83–84°. (Found: C, 51·2; H, 6·1; N, 14·6. $C_{12}H_{17}O_5N_3$ requires C, 50·85; H, 6·05; N, 14·8%.) Described as an oil by Grassmann *et al.* (1953).

*N*-DNP-DL-Phenylalaninol (benzene–*cyclo*hexane), m.p. 122–123°. (Found: C, 57·1; H, 4·9. Calc. for $C_{15}H_{15}O_5N_3$: C, 56·8; H, 4·8%.) (Cf. Grassmann *et al.* 1953; Jatzkewitz & Tam, 1954.)

*N*-DNP-DL-Serinol (chloroform), m.p. 133°. (Found: C, 42·4; H, 4·3; N, 16·1. Calc. for $C_9H_{11}O_6N_3$: C, 42·0; H, 4·3; N, 16·35%.) (Cf. Jatzkewitz & Tam, 1954; Waldkötter, 1938.)

*N*-DNP-DL-Threoninol (benzene containing a few drops of ethanol), m.p. 126–127°. (Found: C, 44·7; H, 4·7. $C_{10}H_{13}O_6N_3$ requires C, 44·3; H, 4·8%.)

*N*-DNP-DL-Glutamidiol and *N*-DNP-DL-prolinol. The crude glutamidiol used contained prolinol as pointed out by Karrer *et al.* (1948). The crude product from the reaction with DNBS was subjected to column chromatography using solvent system no. 2. Two main bands were obtained: *R*, 1·25 and *R*, 0·13. The latter was assumed to be DNP-glutamidiol by comparison with other DNP-aminodiols. The band *R*, 1·25 was purified further in system no. 3 to give a substance *R*, 1·04. Comparison with other DNP-amino alcohols in systems nos. 3, 4 and 5 indicated that this was probably DNP-prolinol. Neither of these DNP derivatives could be crystallized.

The following DNP derivatives were prepared, using borax solution as reaction medium, by the technique described later:

*N*-DNP-DL-Aspartidiol. This substance was obtained as an oil. It was not easily distinguished from DNP-glutamidiol in any of the solvent systems given in Table 2.

*N*-DNP- and *N*,*N'*-bis-DNP-DL-lysinol. Two products were obtained from the reaction and had *R*, 0·21 and *R*, 0·9 in system no. 3. These were assumed to be the DNP- and bis-DNP derivatives respectively. Neither was obtained crystalline.

*N*-DNP-DL-Tyrosinol (chloroform containing a few drops of ethanol), m.p. 186–187°. (Found: N, 12·6. $C_{15}H_{15}O_6N_3$ requires N, 12·6%.)

*N*-DNP-*O*-Methyl-DL-tyrosinol (benzene–light petroleum, b.p. 40–60°), m.p. 138°. (Found: N, 12·1. $C_{16}H_{17}O_6N_3$ requires N, 12·1%.)

### *Preparation and use of the columns*

Hyflo Super-Cel (Johns–Manville Co., London) was made into a thin cream with 6N-HCl and the mixture allowed to stand overnight. It was then filtered and treated twice more with 6N-HCl in the same way. The solid was finally washed free of chloride and dried at 100°. The dried solid was then ignited at 600° for 8 hr. The columns were glass tubes about 35 cm. long and internal diameter 12 mm. The Hyflo Super-Cel (6 g.) was mixed with the stationary phase (3 g.) and poured, in the form of a slurry with the moving phase, into the tube. The column was packed with a steel plunger as described by Howard & Martin (1950). The solvent systems described in Table 2 gave very stable columns which could be used many times. When not in use they were preserved in stoppered glass tubes long enough for the column to be completely enclosed together with sufficient moving phase to cover the solid support both inside and outside the column.

The columns were used under slight positive pressure (3–5 cm. Hg) and were allowed to become just dry before application of the sample in 1 ml. or less of the moving phase. This was then followed by two 0·2 ml. portions of moving phase before filling the upper part of the tube. When dealing with an unknown mixture it was convenient to use first solvent system no. 1 in which all but the aminodiol derivatives ran very fast. Identification of the constituents of the fast-moving material was carried out in systems nos. 3, 4 or 5. The separation of DNP-alaninol and dinitroaniline proved to be difficult. System no. 6 was developed for this purpose. A feature of interest is the great difference in *R* value between DNP-serinol and other DNP-aminodiols in system no. 7. This may be due to interaction between the OH groups of glycerol and the serinol derivative.

The solvent was removed from the eluates by evaporation on the water pump at 30–40°. The residue was dissolved in chloroform and its light absorption measured in the Hilger Uvispek spectrophotometer at 350 mμ. A standard curve was constructed for ethanolamine and alaninol; other DNP derivatives were estimated by reference to the alaninol curve, due allowance being made for molecular weight; it was assumed that the error involved would be small. In some cases an appreciable residue of glycol remained after removal of the mobile phase from the eluate. This did not interfere with the absorption measurement but if it was necessary to apply the residue to another column the glycol was removed by heating at 50°/0·01 mm. Hg for a few minutes.

### *Isolation of amino alcohols from hydrolysates*

The technique used for the preparation of DNP-amino alcohols, although satisfactory when the amino alcohols were fairly pure, gave much lower yields in the presence of a protein hydrolysate. DNP-Ethanolamine was recovered in only 54% yield when a mixture of one part of ethanolamine and 40 parts by weight of a silk hydrolysate was heated with DNBS and N-$Na_2CO_3$ solution at 100° for 1 hr. In view of the high yields of DNP-derivatives obtained from pure amino alcohols the low yield was considered to be due to decomposition of DNP-ethanolamine rather than to incomplete reaction. Amino alcohols were found to react rather slowly with DNBS in the presence of $Na_2CO_3$ at 30 and 40°; when NaOH was used in place of $Na_2CO_3$ extensive destruction of the DNP derivative took place. It was then found that a 95% yield of DNP-alaninol was obtained by heating alaninol with excess of DNBS in the presence of borax solution at 95° for 17–20 hr. This method was also effective when amino acids were present.

Silk fibroin (20 mg.) was hydrolysed in the usual way and the hydrolysate evaporated to remove excess HCl. The residue was dissolved in water (1 ml.) and 0·5 mg. each of the oxalates of ethanolamine, alaninol and serinol were then added. To this solution was then added N-NaOH until the pH was 9·5–10·0 (indicator paper). The mixture was washed into a tube containing borax (1 g.) and DNBS (200 mg.); the volume of liquid was then about 5 ml. The tube was sealed and heated at 95° for 20 hr., with occasional shaking at first until the borax had dissolved. At the end of the reaction water (10 ml.) was then added and the DNP-amino alcohols were extracted with ether. The ether extract was evaporated to dryness and applied to a Hyflo Super-Cel column using solvent system no. 1, which separated DNP-serinol from the DNP derivatives of ethanolamine, alaninol and ammonia, which formed a single fast-moving band. This mixture was then applied to a column using system no. 3 in which DNP-ethanolamine was separated from dinitroaniline and DNP-alaninol. The

last two substances were separated in system no. 6. The yields of DNP-serinol, DNP-ethanolamine and DNP-alaninol were 80, 89 and 85% respectively.

It was found convenient to use the apparatus shown in Fig. 1 for the ether extraction of DNP-amino alcohols after the DNBS reaction. The walls of the washing limb *C*, and of the extraction limb *B* were thickened close to the lower side-arms so as to provide narrow orifices through which ether was forced by hydrostatic pressure forming fine jets and creating considerable turbulence of the liquid. After

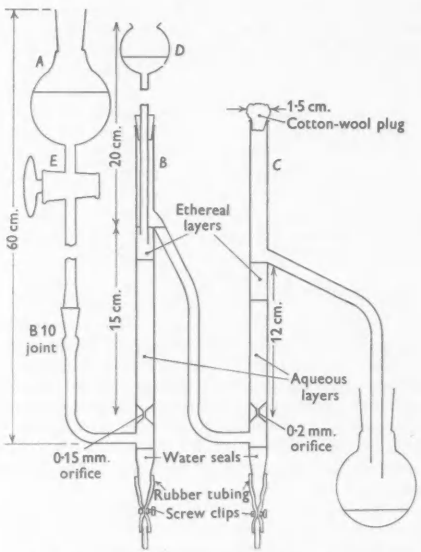



Fig. 1. Diagram of extraction apparatus.

proceeding for a short distance the liquid streams broke up into fine droplets which travelled to the surface of the aqueous layer before coalescing. At the commencement of an extraction the drain-cocks attached to *B* and *C* were closed and sealed with water. Ether was poured into the reservoir *A* and allowed to flow into limb *B* until the orifice was just covered with ether. Tap *E* was then closed and the solution to be extracted washed into limb *B*, the final volume being not more than 15 ml. The thistle funnel *D* was then fixed in position and tap *E* opened. As soon as the ether extract from *B* had reached the orifice in limb *C*, about 12 ml. of water were quickly added to this limb and *D* was half-filled with fresh ether. The progress of the extraction was controlled by tap *E*, on which no lubricant other than the solvent was used. Extraction of DNP-amino alcohols was complete after 100 ml. of ether had been passed through the apparatus; this required about 10 min. In the case of mixtures containing a large quantity of dinitroaniline, which has a much higher water solubility than most DNP-amino alcohols, the aqueous layer in limb *C* was coloured deep yellow and the washed ether layer remained pale yellow even after this volume of ether had been passed through. Control experiments showed, however, that the extraction could safely be interrupted at this point.

### *Action of* $LiBH_4$ *on the formyl peptides obtained from silk fibroin*

The peptide mixture (100 mg.) (Elliott, 1953) was dissolved in water (10 ml.) and methanol (20 ml.) added to the soln. Ethereal diazomethane soln. was then added in small portions with shaking until sufficient was present in excess to maintain a yellow colour for at least 2 min. After standing for 0·5 hr. the excess of reagent was destroyed with a drop of acetic acid and the solution evaporated under reduced pressure until the ether and methanol had been removed. The aqueous residue was then dried in the frozen state. The above operations and the reduction described below were carried out in a thick-walled cylindrical flask of approx. 6 cm. diam. and 8 cm. length, excluding the neck. To the residue was then added dry THF (25 ml.), $LiBH_4$ (25 mg.) and a number of small glass beads (occupying a volume of about 10 ml.). In the reaction at room temperature the flask was mounted in a horizontal position and slowly rotated, but in the experiments at 75° the flask was mounted in an upright position in an oil bath, and agitation of the contents was obtained by means of a stout glass stirrer. At the end of the reduction excess of reagent was decomposed by addition of a few drops of 3 N-HCl and the solvent removed under reduced pressure. A sticky residue remained which was removed from the flask by boiling for a few min. with several portions of 6 N-HCl and decanting from the glass beads. The acid extracts were evaporated to small bulk and then heated in a sealed tube at 100° for 16 hr. The hydrolysate was treated with DNBS (1 g.) in the presence of borax (2·5 g.), exactly as described for the silk fibroin hydrolysate.

The reduction of the unesterified peptide mixture was carried out in a similar fashion.

### *Action of* $LiBH_4$ *on lysozyme*

The reduction was carried out exactly as for the peptides from silk fibroin. The lysozyme was prepared from egg-white by the method of Fevold & Alderton (1949) and was recrystallized four times.

To identify the *N*-terminal residues liberated during the reduction a separate experiment was carried out and the excess of $LiBH_4$ decomposed with a few drops of glacial acetic acid. The solvent was removed under reduced pressure and the residue dried in a vacuum desiccator over KOH. The residue was treated with 10% (w/v) $NaHCO_3$ (10 ml.), ethanol (10 ml.) and fluorodinitrobenzene (0·1 ml.), and the mixture vigorously sitrred for 4 hr. Excess of 6 N-HCl was then added and the soln. evaporated almost to dryness. The sludge of DNP-peptides was hydrolysed by refluxing 16 hr. with 6 N-HCl and the DNP-amino acids were isolated in the usual way. Dinitrophenol was removed from the DNP-amino acid mixture by the method of Isherwood & Cruickshank (1952). Chromatography of the mixture was carried out on paper buffered at pH 6, using *tert.*-amyl alcohol as solvent (Blackburn & Lowther, 1951). Six spots of about equal intensity were obtained. These had $R_F$ values identical with those of the DNP derivatives of the following groups of amino acids: Gly and/or Ser, Asp and/or Glu and/or $CyS_2$, Ala and/or Met, Thr and/or Arg, Val, Leu.

*Attempts to use* $LiBH_4$ *in aqueous solution*

It was found that gradual addition of a solution of $LiBH_4$ in THF to water produced at first a vigorous effervescence and the pH of the solution rose rapidly to 9·5. Decomposition of the reagent then took place much more slowly.

Methyl hippurate (19·3 mg.; 0·1 m-mole) was dissolved in a mixture of methanol (1 ml.) and water (19 ml.) and to it was added a 0·1 M soln. of $LiBH_4$ in THF (2 ml.) and the mixture allowed to stand at room temp. for 3 hr. An additional portion of 2 ml. of reagent was then added, followed by two more additions of 2 ml. after 19 and 26 hr. from the beginning of the reaction. After 28 hr. had elapsed the product was hydrolysed and ethanolamine estimated as the DNP derivative as previously described. The yield was 42% of the theoretical. No improvement in the yield was obtained in the presence of cobalt sulphate, and in the presence of boric acid it was considerably decreased. These reagents are known to catalyse the decomposition of $LiBH_4$ in aqueous solution (Schlesinger, 1944) and it was hoped that they might also catalyse the reduction.

Lysozyme was esterified by two different procedures: first, by the action of diazomethane as described above for the formyl peptides from silk fibroin, and secondly by methanolic hydrogen chloride as described by Fraenkel-Conrat & Olcott (1945). The products were treated in aqueous methanol with $LiBH_4$ in a manner similar to that used for methyl hippurate, but in neither case was an appreciable quantity of DNP-leucinol obtained from the hydrolysates.

## DISCUSSION

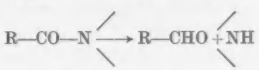
Some of the DNP-amino alcohols described in the experimental section were not obtained crystalline but the *R* values of these products agreed well with expectations. It was not possible to achieve separation of DNP-leucinol from DNP-isoleucinol; the separations of DNP-aspartidiol from DNP-glutamidiol and of DNP-phenylalaninol from DNP-*O*-methyltyrosinol were not satisfactory. The last compound would probably be produced from a tyrosine end-group if diazomethane were used as an esterifying agent. Under the conditions used for isolation from hydrolysates lysinol appeared to give a mixture of the singly and doubly substituted DNP derivatives. The clarification of these points was not pursued in view of the unsatisfactory results obtained with lithium borohydride.

It is now known that two different side reactions can occur at the peptide bond during reduction of peptides or their esters by mixed metallohydrides:

$$—CO—NH— \longrightarrow —CH_2—NH— \quad (1)$$

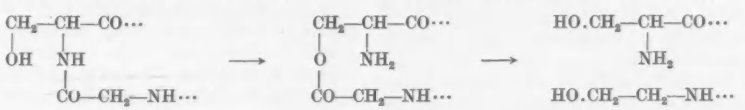
$$—CO—NH— \longrightarrow —CH_2.OH + NH_2— \quad (2)$$

Reaction (1) was observed by Karrer & Nicolaus (1952) in reductions of peptides by lithium aluminium hydride under vigorous conditions. The results of the present work show that it can take place even under relatively mild conditions. It is likely to occur in amides generally (Gaylord, 1954). Reaction (2) has been observed in amides when treated with either lithium borohydride or lithium aluminium hydride (Wittig & Hornberger, 1952; Snyder & Putnam, 1954) and it appears that the nature of the substituents attached to the amide nitrogen atom determines the ease with which fission occurs. In some cases fission can be brought about under very mild conditions and the reaction can be used for the preparation of aldehydes (Wittig & Hornberger, 1952):



This reaction is analogous to scheme (2) above. Presumably the peptide bond is cleaved, yielding an aldehyde, which is then further reduced to a carbinol.

It has been shown that ethanolamine is produced from insulin by the action of lithium aluminium hydride (Fromageot & Jutisz, 1953) and of lithium borohydride (Chibnall & Rees, 1953), although glycine is not a *C*-terminal amino acid in this protein. The latter authors produced evidence to show that this may have been due to acyl migration at the glycylserine sequence, followed by reductive cleavage of the ester group:



In view of the number of different *N*-terminal residues liberated, a specific fission of this nature was unlikely to have occurred in lysozyme. It was found, however, that bonds involving the carboxyl groups of glycine or alanine were more susceptible to reduction than other peptide bonds, but this is probably due to the small size of the side chains.

As a result of this work it appears that, with the technique available at present, lithium borohydride cannot be used for the determination of the *C*-terminal residues of large peptides and proteins. This is due to the vigorous conditions which must be employed on account of the very low solubility of these large molecules in organic solvents. On the other hand, satisfactory results can be obtained on small peptides which are sufficiently soluble to allow reduction to take place at a reasonable rate of low temperature. Recently, Grassmann, Endres & Steber (1954) have suggested that procollagen contains ester bonds because it yields amino alcohols on treatment with lithium borohydride at the boiling point of THF. In a subsequent paper

Grassmann, Hörmann & Endres (1955) described the reduction of insulin and several peptides. Reductive cleavage of peptide bonds in esterified peptides was not observed under the conditions they used, although these were, apparently, more vigorous than the conditions used in the present work. On the other hand, alaninol was obtained from insulin itself in a yield of about 20 % of that obtained from the esterified protein. In view of the small degree of reduction of hippuric acid observed in the present investigation, and of the free peptides studied by Grassmann *et al.* (1955) it seems likely that free carboxyl groups of proteins would be reduced to a very small extent by lithium borohydride. It is therefore possible that part of this alaninol arose by fission of the peptide chain and not from the *C*-terminal residue of insulin.

## SUMMARY

1. Lithium aluminium hydride and lithium borohydride were compared as reducing reagents for the *C*-terminal residues of benzoylated peptide esters. The latter reagent gave satisfactory results provided that the reaction was carried out at 0°.

2. Attempts to devise a stepwise degradation of peptides by means of the *N*, *O*-acyl migration reaction on the reduced peptide derivatives were unsuccessful.

3. A new method for the preparation of the DNP derivatives of amino alcohols is described. This was used for the quantitative isolation of amino alcohols from hydrolysates of proteins and peptides after reduction.

4. The value of lithium borohydride as a reagent for the determination of the *C*-terminal residues of large peptides and proteins was critically assessed. Owing to the low solubility of these substances in organic solvents, it was necessary to use more vigorous conditions than those required for reduction of simple peptides. Reductive cleavage of peptide bonds then took place simultaneously with the reduction of *C*-terminal residues. Correct identification of *C*-terminal residues with this reagent is therefore not possible under the conditions at present available.

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# Glycine Uptake by the Proteins of the Rat Pancreatic Juice

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Although extensive research has been done on the morphology and cytophysiology of cell secretion (see reviews by Hirsch, 1931, 1939, 1955) few data exist on the chemical phenomena related to this process.

As a continuation of a series of studies on the biochemistry of cell secretion (Junqueira, 1951; Rabinovitch *et al.* 1952; Fernandes & Junqueira, 1953, 1955) experiments were performed in order to obtain data on the rate at which this process occurs.

The pancreas is a convenient organ to work with, for besides actively secreting proteins its activity can be stimulated or depressed by pharmacological agents. Furthermore, up to the present time no blood proteins have to our knowledge been proved to permeate this gland and to be excreted with pancreatic juice; if this in fact occurred, however, it would complicate the interpretation of the results obtained.

We present here the results obtained by studying the rate of appearance of radioactivity in the rat pancreatic-juice proteins after intravenous injections of radioactive glycine and radioactive plasma proteins. Furthermore, the protein-bound and 'non-protein' radioactivity of blood plasma was studied during the experiments.

## MATERIAL AND METHODS

Adult male Wistar rats weighing from 250 to 380 g. (average 306 g.) were used throughout. They were starved 24 hr. previously but water was given *ad lib.* Anaesthesia was performed by injecting intraperitoneally 8·0 mg. of Numal (Roche) per 100 g. body weight.

### *Radioactivity in pancreatic juice after injection of glycine*

In these experiments a total of nine rats were used. Pancreatic juice was collected in 10 $\mu$l. glass pipettes from a polyethylene cannula introduced into the common biliary and pancreatic duct 2–3 mm. above its entrance into the duodenal wall. To avoid contamination with bile a high ligature of the biliary duct was performed 5 mm. below its emergence from the liver. A continuous flow of pancreatic juice was promoted by intravenous injection into the femoral vein of 0·7 unit of secretin and 1·0 $\mu$g. of carbamoylcholine (cerbachol) dissolved in 0·2 ml. saline per rat. This stimulus was repeated every hour up to the end of the experiment at half of the initial dose.

Approximately 5 min. after the first stimulus 3·3 $\mu$c/100 g. body weight of [1-$^{14}$C]glycine (specific activity, 32·5 $\mu$c/mg., supplied by the Radiochemical Centre, Amersham) were injected at the rate of 1 ml./kg. body weight into the femoral vein.

*Radioactivity assays.* To assay the radioactivity of the pancreatic juice, 10 $\mu$l. samples of pancreatic juice were periodically collected, mixed with 2 $\mu$l. of 0·1 N-NaOH and spread out evenly on a stainless-steel planchet (3·11 sq.cm.) and immediately dried by heating at 40°. With this technique an average of fifteen samples of pancreatic juice and seven of plasma could be studied in each experiment. Radioactivity determinations were performed on a Tracerlab gas-flow counter. Every sample was counted three times and the counts agreed to within 3%. Samples were corrected for self-absorption by using a standard curve obtained from measurements of radioactive pancreatic-juice proteins.

*Protein estimations.* The amount of protein present in the samples was assayed by the method described by Lowry, Rosebrough, Farr & Randall (1951), previously checked by Kjeldahl nitrogen determinations. The average amount of protein in the samples was 261·9 $\mu$g., with a range of 25·5–709·4.

*Separation of protein-bound and non-protein radioactivity.* In order to separate protein-bound radioactivity from radioactivity bound to smaller molecules (non-protein radioactivity) the following procedure was adopted. The planchets were immersed during 10 min. each in absolute ethanol, 0·5% collodion in ethanol–ether (1:1, by vol.), 70% (w/v) ethanol, 30% (w/v) ethanol and distilled water. They were then transferred to 10% (w/v) trichloroacetic acid for 3 hr. at 37° and afterwards immersed in distilled water for 12 hr. with five changes. Preliminary experiments showed that when radioactive glycine is added to non-radio-

active pancreatic juice and extracted as described, all the radioactivity is lost after only 2 hr. of immersion in water. The planchets were then washed with 35% and 70% (w/v) ethanol, absolute ethanol and six changes of ethanol–ether (1:1 by vol.) (10 min. each) in order to dissolve the collodion, and dried at 37°.

### *Radioactivity in pancreatic juice after injection of radioactive plasma proteins*

Three rats were injected with radioactive plasma proteins prepared by ammonium sulphate precipitation of plasma obtained from oxalated blood of animals injected intravenously 6 hr. previously with 3·3 $\mu$c glycine per 100 g. body weight. These proteins were redissolved in saline and had an average activity of 494 counts/mg. protein. Each animal received the equivalent of 4 ml. plasma.

The pancreatic juice was collected and assayed for radioactivity from animals stimulated by secretin and carbamoylcholine as described above.

### *Radioactivity of the plasma of animals injected with glycine*

In six of the animals used for the pancreatic-juice experiments blood was collected hourly in oxalated tubes and 10 $\mu$l. samples of plasma were assayed for free and protein-bound radioactivity as described for the pancreatic juice.

In three other animals blood was collected at short intervals soon after the glycine injection, in order to study the rate of disappearance of the non-protein radioactivity.

## RESULTS

### *Incorporation of radioactivity into the pancreatic-juice proteins*

The results obtained are presented in Table 1 and the curve of Fig. 1 represents the average result of 141 samples obtained from nine animals. It may be seen that the maximum radioactivity is attained $2\frac{3}{4}$ hr. after injection of [$^{14}$C]glycine. Appreciable radioactivity appears in the pancreatic-juice protein only 50 min. after the injection. Fig. 1 also shows the rate of pancreatic-juice production under our experimental conditions. Although these and other results related to the physiology of the rat pancreas are to be published later, this curve is presented here in order to stress the point that maximum radioactivity appears only almost 3 hr. after glycine injection, although the action of the first injection of secretin and carbamoylcholine on pancreatic secretion was quite prompt. It is interesting to observe the similarity of our curve to the one obtained by Askonas, Campbell & Work (1954) for the incorporation of methionine into goat-milk proteins.

Analysis of the radioactivity measurements of samples before and after ethanol, ether, trichloroacetic acid and water extractions showed no constant and significant difference, suggesting that practically all the radioactivity in the pancreatic juice was bound to proteins.

Table 1. *Radioactivity of rat pancreatic-juice proteins after injection of carbamoylcholine–secretin and* [1-$^{14}$C]*glycine*

Results expressed in counts/min./mg. protein.

| Time after glycine injection (min.) | Number of rats ($n$) | Average counts ($\bar{x}$) | Standard deviation ($s$) | Confidence interval* 95% $\bar{x}\pm t\frac{s}{\sqrt{n}}$ |
|---|---|---|---|---|
| 0–20 | 9 | 11 | 22 | 11±17 |
| 20–40 | 9 | 43 | 87 | 43±67 |
| 40–60 | 8 | 352 | 276 | 352±231 |
| 60–80 | 9 | 1841 | 1855 | 1841±1426 |
| 80–100 | 9 | 6356 | 7364 | 6356±5660 |
| 100–120 | 8 | 7861 | 6536 | 7861±5481 |
| 120–140 | 9 | 9990 | 6456 | 9990±4962 |
| 140–160 | 9 | 9407 | 4699 | 9407±3612 |
| 160–180 | 9 | 11486 | 5960 | 11486±4225 |
| 180–200 | 9 | 10791 | 3261 | 10791±2507 |
| 200–220 | 9 | 10439 | 5747 | 10439±4417 |
| 220–240 | 8 | 9387 | 2675 | 9387±2235 |
| 240–260 | 9 | 8203 | 2144 | 8203±1648 |
| 260–280 | 6 | 9198 | 2866 | 9198±3007 |
| 280–300 | 5 | 6934 | 3355 | 6934±4158 |
| 300–320 | 5 | 7438 | 2489 | 7438±3085 |
| 320–340 | 4 | 5096 | 843 | 5096±1341 |
| 340–360 | 2 | 4507 | 1518 | 4507±13641 |
| 360–380 | 3 | 5001 | 842 | 5001±2092 |
| 380–400 | 2 | 4759 | 1389 | 4759±12481 |
| 400–420 | 2 | 3300 | 1131 | 3300±10163 |
| 420–440 | 2 | 2814 | 733 | 2814±6587 |

* $t$ = the critical value of the statistic '$t$' (Student's table) at the 5% level with $n-1$ degrees of freedom.

A possible source of error in the evaluation of the rate of appearance of radioactivity in the proteins of the pancreatic juice might be due to the time taken by these proteins to get from the cells to the distal end of the pancreatic duct. Inorganic phosphate is a normal component of pancreatic juice (La Barre, 1953) and the rate of its appearance might indicate the time taken by the juice to pass through the pancreatic duct. Sodium [$^{32}$P]phosphate (60 $\mu$C, supplied by Abbott Laboratories) was therefore injected into three rats and the pancreatic juice collected and assayed for radioactivity as described above. In all rats injected with sodium [$^{32}$P]phosphate a distinct radioactivity (300–400 counts/min.) could be observed 3 min. after the injection; the time after which the peak of $^{32}$P radioactivity was reached was approximately 1 hr. These animals were studied for 2½ hr. and a gradual decrease of this radioactivity was observed.

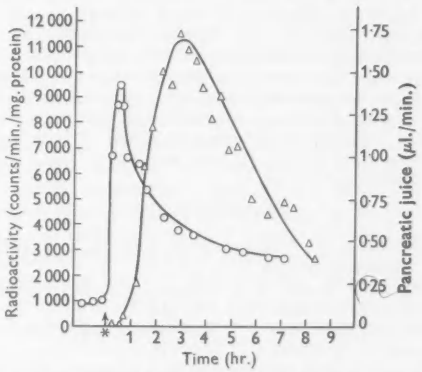


Fig. 1. Appearance of radioactivity in the rat pancreatic-juice proteins after injection of 3·3 $\mu$C [1-$^{14}$C]glycine and after secretin–carbamoylcholine stimulation. △, each point is the average radioactivity of samples from nine animals; ○, rate of pancreatic juice flow. The asterisk indicates the time of the first stimulation and the arrow the time at which glycine was injected.

This result suggests that no appreciable time is lost by the juice proteins to pass from the pancreatic cells to the main excretory duct. This point of view is corroborated by the results of Ball, Tucker, Solomon & Vennesland (1941), who described the prompt appearance of intravenously injected radioactive bicarbonate in pancreatic juice.

*Radioactivity of pancreatic juice after injection of radioactive plasma proteins*

In the three rats studied no radioactivity above background was observed in twenty-two samples of pancreatic juice collected during 6 hr.

*Radioactivity in plasma of animals injected with [$^{14}$C]glycine*

The appearance of radioactivity in the plasma after glycine injection is shown in Fig. 2. The protein-bound radioactivity reaches a maximum after 4 hr. The non-protein radioactivity, however, decreases rapidly, reaching a very low level after 1 hr. and disappearing after 8 hr.

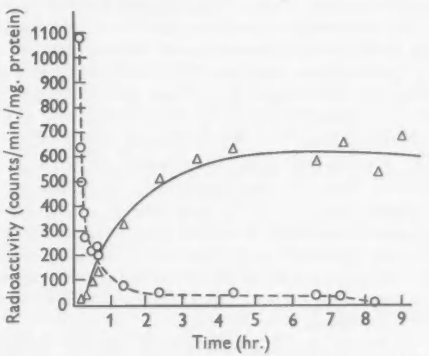


Fig. 2. Appearance of radioactivity in the blood plasma proteins of the rat after injection of [$^{14}$C]glycine. △, Each point is the average radioactivity of samples from six rats; ○, non-protein plasma, each point is the average radioactivity from six rats.

## DISCUSSION

The protein-bound radioactivity in pancreatic juice reaches its maximum about 3 hr. after the injection of [1-$^{14}$C]glycine (Fig. 1) although the first injection of secretin and carbamoylcholine stimulates maximum flow of juice much earlier. The slope of the radioactivity curve for the juice proteins gives an idea of the rate of pancreatic secretion and consequently the time necessary for the synthesis of the proteins of the pancreatic juice. It is of interest to compare these results with those obtained by other authors who studied pancreas secretion even though different methods and species were used. Hirsch (1932) described a complete restitution of secretory granules in mouse pancreas, 12–14 hr. after pilocarpine administration. Secretory granules, however, appeared after about 3 hr. van Weel & Engel (1938), Daly & Mirsky (1953) and Sesso (personal communication) obtained in pilocarpine-stimulated mice resynthesis of carboxypeptidase and amylase activity respectively 12 and 7 hr. after the injection. Mathematical calculations of Langstroth, McRae & Komarov (1939), based on the rate of protein secretion by the dog pancreas, indicate that the

time necessary for the restoration of secretory granules from a previously depleted gland would be about 3 hr.

Our results on the incorporation of glycine into plasma proteins agree with those of Friedberg, Tarver & Greenberg (1948), and the curve of disappearance of non-protein radioactivity from blood plasma presented by us agrees with the data obtained by Miller, Bly & Bale (1954).

The fact that no radioactivity was detected in the pancreatic juice of rats injected with radioactive plasma proteins shows that these proteins do not contribute appreciably to the formation of pancreatic-juice proteins. This conclusion is supported by the observation that after injection of labelled glycine the peak in the radioactivity of the pancreatic-juice proteins occurred before that of the plasma proteins. If plasma proteins were utilized for the synthesis of pancreatic-juice proteins, the peak in the radioactivity of the latter would be expected to occur some time after the plasma proteins had reached maximum radioactivity and would be less than that of the plasma proteins (Zilversmit, Entenman & Fishler, 1943; Hevesy, 1948).

Although direct utilization of plasma proteins for milk-protein synthesis has been suggested by Graham, Peterson, Houchin & Turner (1937), more recent observations by Askonas *et al.* (1954) do not support this view, and the present work suggests that pancreatic-juice proteins are also not synthesized directly from plasma proteins.

The almost complete absence of radioactivity in the pancreatic-juice proteins in the first 50 min. after injection of glycine suggests that there is no appreciable interchange between free glycine and protein-bound glycine, since this would lead to an earlier appearance of radioactivity in the pancreatic-juice proteins.

The present observations thus suggest that the proteins of rat pancreatic juice are derived mainly from free amino acids. Recently Askonas *et al.* (1954) came to a similar conclusion about the formation of goat-milk proteins.

## SUMMARY

1. Rats were injected with [1-$^{14}$C]glycine or with radioactive plasma proteins, and the appearance of radioactivity in the pancreatic juice proteins was followed.

2. Maximum radioactivity was observed 2¾ hr. after the injection of [$^{14}$C]glycine.

3. No appreciable radioactivity was observed in the first 50 min. after the glycine injection.

4. The appearance of protein-bound and non-protein radioactivity in the plasma of animals injected with glycine was also followed.

5. No radioactivity could be detected in the pancreatic juice of animals injected with radioactive plasma proteins.

The significance of these results is discussed.

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# The Oxidation of Manganese by Illuminated Chloroplast Preparations

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It has long been known that manganese is an essential micronutrient of plants. Although the precise functions of manganese are unknown there is evidence that it may be concerned in chlorophyll synthesis, photosynthesis, respiration and nitrate assimilation. Decreased photosynthesis under conditions of manganese deficiency was originally attributed to the diminished chlorophyll content, but more recent investigations suggest the possibility of a direct effect of manganese on photosynthesis. The evidence, which is conflicting, is reviewed by Ruck & Bolas (1954). Arnon (1954) and Gerretsen (1937, 1949, 1950) obtained evidence in favour of a direct effect of manganese on photosynthesis. Gerretsen (1950) concluded from potentiometric measurements that $H_2O_2$ was formed when chloroplast preparations were illuminated in the presence of $Mn^{2+}$. It was suggested that $Mn^{2+}$ functions as an activator of oxygen and that a photosensitive oxidation–reduction system is present in chloroplasts in which there is equilibrium between $Mn^{2+}$ and $Mn^{3+}$ or $Mn^{4+}$, this equilibrium moving in the direction of oxidation during illumination. Mehler (1951*a*, *b*) demonstrated the formation of $H_2O_2$ by illuminated chloroplast suspensions by showing that addition of catalase to such suspensions resulted in the coupled oxidation of added ethanol. Kenten & Mann (1950) showed that peroxidase systems catalyse the oxidation of $Mn^{2+}$. If chloroplast preparations contain peroxidase the hydrogen peroxide formed on illumination might be used for the oxidation of $Mn^{2+}$. The present work was undertaken to find out whether illuminated chloroplast preparations oxidize $Mn^{2+}$ and whether such oxidation is due to peroxidase systems.

## MATERIALS AND METHODS

*Plant material.* Most of the plants used were glasshouse-grown in a John Innes potting compost.

*Leaf extracts.* These were made from leaves of tobacco (*Nicotiana tabacum* L.), spinach (*Beta vulgaris* L.), pea (*Pisum sativum* L.) and broad bean (*Vicia faba* L.). The leaves (20 g.) were ground with sand and 100 ml. 0·04 M-phosphate pH 7 at 0° in a chilled mortar. The macerate was squeezed by hand through strong cotton cloth into a chilled tube, centrifuged for 2 min. at 2000 r.p.m. (900 *g*) and the precipitate discarded. The values given for *g* refer to the centrifugal force at the bottom of the centrifuge tubes.

*Chloroplast preparations.* The leaf extract (100 ml.) was centrifuged at 10000 r.p.m. (11000 *g*) for 15 min. in an MSE (Measuring and Scientific Equipment Ltd.) angle-head centrifuge at 0–4°. The precipitate was suspended in 100 ml. 0·04 M phosphate, pH 7, at 0° and centrifuged again at 10 000 r.p.m. for 15 min. at 0–4°. The precipitate of chloroplast material was suspended in 0·04 M phosphate, pH 7, to a convenient volume and stored at 0°.

*Peroxidase.* This was prepared from horseradish roots by the method of Kenten & Mann (1954). The *Purpurogallinzahl* (P.Z.) (Willstätter & Stoll, 1918) of the preparation, i.e. mg. purpurogallin formed by 1 mg. enzyme preparation in 5 min. from pyrogallol and $H_2O_2$ under fixed conditions was estimated by the method of Keilin & Hartree (1951) using an EEL (Evans Electroselenium Ltd.) colorimeter with Ilford Bright Spectrum Blue filter 622 (maximum transmission at $\lambda$ 465 m$\mu$.). The preparation of P.Z. 1040 used was about 90% peroxidase.

*Catalase.* This was prepared from ox liver by the method of Sumner & Dounce (1937) and the activity of the preparation was estimated as described previously (Kenten & Mann, 1952*b*). The *Katalasefähigkeit* (Kat.f.) was 11000, suggesting a purity of about 20%. From the equation of Chance & Herbert (1950) that Kat.f. $= 520\, k_1'/M$, where $M$ is the molecular weight of catalase (230 000), the velocity constant of the preparation, $k_1 = 4{\cdot}9 \times 10^6\, M^{-1}$ sec.$^{-1}$.

*Manganipyrophosphate solution.* This was prepared by the procedure of Heintze & Mann (1949), which depends on the reaction

$$Mn^{2+} + MnO_2 + 4H^+ \rightleftharpoons 2Mn^{3+} + 2H_2O. \qquad (1)$$

Excess of hydrated $MnO_2$ was added to solutions of $MnSO_4$ in 0·1 M pyrophosphate at pH 7. The mixtures were allowed to stand with occasional shaking at room temperature for 18 hr. The residual $MnO_2$ was then removed by centrifuging. The manganipyrophosphate in the supernatant solutions was estimated by titration against standard $FeSO_4,(NH_4)_2SO_4$.

Solutions of manganipyrophosphate in 0·1 M pyrophosphate at pH 7 absorb ultraviolet light strongly, with an absorption maximum at 258 m$\mu$. (Fig. 2). At this wavelength such solutions of manganipyrophosphate obey Beer's law at least over the range of concentration of 2–7 $\mu$g. $Mn^{2+}$/ml. (Fig. 1). Manganipyrophosphate in experimental reaction mixtures was estimated by measuring the absorption at 258 m$\mu$., or manometrically with hydrazine sulphate according to the method of Kenten & Mann (1949). The results of Table 1 show a close agreement between the two methods of estimation.

*Spectrophotometry.* Absorption data were determined by the use of a Unicam model SP. 500 quartz spectrophotometer.

*Chlorophyll.* This was estimated spectrophotometrically by the method of Arnon (1949).

*Illumination.* An Osram Daylight fluorescent tube was the source of light.

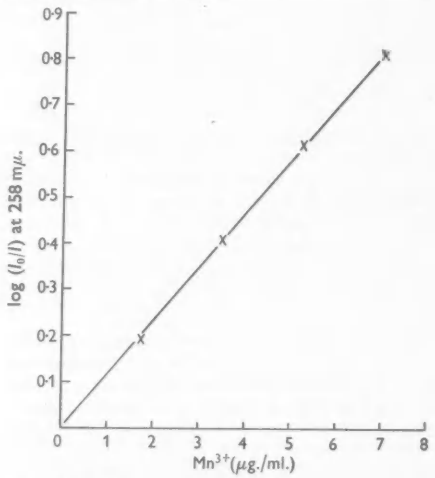


Fig. 1. Effect of manganipyrophosphate concentration on the absorption of ultraviolet light at 258 mμ. 0·1 M-pyrophosphate, pH 7.

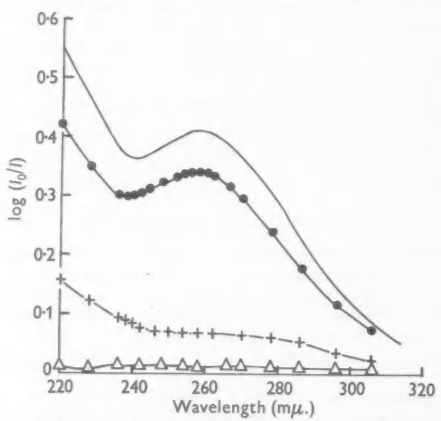


Fig. 2. Spectrophotometric evidence for the oxidation of $Mn^{2+}$ by illuminated chloroplast preparations. Samples of a tobacco chloroplast preparation were incubated with and without $MnSO_4$ in light (100 ft.c.) and in darkness for 3 hr. After clarification by centrifuging the ultraviolet absorption curves of the solutions were determined by using the mixture incubated in darkness without added $MnSO_4$ in the blank cell. ●, in light with $MnSO_4$; +, in darkness with $MnSO_4$; △, in light without $MnSO_4$; —, manganipyrophosphate (3·5 μg. $Mn^{3+}$/ml.) in 0·1 M pyrophosphate, pH 7.

Table 1. *Estimation of manganipyrophosphate by the spectrophotometric and manometric methods*

The concentration of the manganipyrophosphate solution (300 μg. $Mn^{3+}$/ml.) was established by titration against standard $FeSO_4,(NH_4)_2SO_4,6H_2O$. For the manometric estimation each vessel contained 2·8 ml. of the undiluted manganipyrophosphate solution. After equilibration 0·2 ml. of saturated hydrazine sulphate solution was added from the side arm. Gas phase, $N_2$; temp. 28°. The $Mn^{3+}$ present was calculated from the $N_2$ outputs after correction for 105% recovery as described by Kenten & Mann (1949). For the spectrophotometric estimation, the absorption at 258 mμ. of suitable dilutions of the manganipyrophosphate solution in 0·1 M pyrophosphate, pH 7, were measured.

| $Mn^{3+}$ present (μg./ml.) | $Mn^{3+}$ found (μg./ml.) | |
|---|---|---|
| | Spectrophotometric method | Manometric method |
| 1·5 | 1·38 | — |
| 3 | 2·95 | — |
| 6 | 6·20 | — |
| 300 | — | 300 |
| 300 | — | 286 |
| 300 | — | 286 |

## EXPERIMENTAL AND RESULTS

### *Oxidation of* $Mn^{2+}$ *by illuminated chloroplast preparations*

*Colorimetric evidence.* Kenten & Mann (1952*a*) showed that when certain plant extracts were incubated with added $Mn^{2+}$ and pyrophosphate at pH 7, the oxidation of $Mn^{2+}$ and the accumulation of the oxidation product in the form of manganipyrophosphate could be demonstrated by means of the intense blue coloration which this compound gives with benzidine. In preliminary experiments in the present work positive tests of this nature were obtained by the use of illuminated leaf extracts and chloroplast preparations in the presence of $Mn^{2+}$ and pyrophosphate pH 7. The tests were made in 50 ml. beakers with reaction mixtures consisting of 2 ml. leaf extract from tobacco or spinach leaves, 3 ml. 0·1 M pyrophosphate (pH 7) with and without 1–2 ml. 0·02 M-$MnSO_4$ and water to 6 ml. The reaction mixtures were illuminated (100 ft.c.) for 2–3 hr. at room temperature. Control reaction mixtures were incubated in the dark. Benzidine acetate (a few drops of 0·2% solution in 10% v/v acetic acid) was then added. Strong blue colorations were obtained with those reaction mixtures containing added $Mn^{2+}$ which had been illuminated, whereas in the absence of added $Mn^{2+}$, or of illumination, negative or only weakly positive tests were obtained. The results suggest the possibility that some of the positive

tests for manganese oxidation obtained by Kenten & Mann (1952*a*) with leaf extracts may have been dependent on illumination. Washed chloroplast suspensions prepared from the leaf extracts also gave strongly positive tests for manganese oxidation in light but not in darkness, while the supernatants from the centrifuged leaf extracts (15 min. at 10 000 r.p.m.) gave negative or, only occasionally, slightly positive tests. The colorimetric tests were negative with extracts or chloroplast preparations which had been heated for 5 min. in a boiling-water bath. The fact that the positive colorimetric tests were due to the accumulation of manganipyrophosphate was subsequently confirmed by the manometric estimation of this compound in the reaction mixtures by using the method of Kenten & Mann (1949). But for much of the work with the chloroplast preparations, particularly where low concentrations of $Mn^{2+}$ were used, the spectrophotometric method of estimation of manganipyrophosphate was found more suitable.

*Spectrophotometric evidence.* The fact that the $Mn^{2+}$ is oxidized by chloroplast preparations in the presence of light was confirmed by experiments in which the accumulated manganipyrophosphate was identified and estimated by the spectrophotometric method. Fig. 2 illustrates the results of one such experiment.

Reaction mixtures consisting of 0·5 ml. washed tobacco chloroplasts (1·67 mg. chlorophyll/ml.), 3 ml. 0·1 M pyrophosphate, pH 7, with and without 0·6 ml. 0·01 M-$MnSO_4$ in a total volume of 6 ml. were incubated either in light (100 ft.c.) or in darkness for 3 hr. Each reaction mixture was then made up to a total volume of 8 ml. and centrifuged at 10 000 r.p.m. for 20 min. The supernatants were diluted with 4 vol. 0·1 M pyrophosphate pH 7, and the ultraviolet absorption curves were determined, using a suitable mixture in the blank cell. The supernatant solution from the illuminated reaction mixture containing added $Mn^{2+}$ gave an absorption curve closely resembling that of manganipyrophosphate. The absorption, like that of manganipyrophosphate, was destroyed within a few minutes by added hydrazine sulphate (0·1 ml. 0·1 M-$N_2H_4,H_2SO_4$/3·5 ml.). From the absorption at 258 m$\mu$. it was calculated that a total of 116 $\mu$g. $Mn^{3+}$ had been formed in this reaction mixture. The amount of added $Mn^{2+}$ (0·6 ml. of 0·01 M soln.) was 330 $\mu$g. The supernatants from the other reaction mixture showed comparatively little absorption of ultraviolet light. Little evidence of $Mn^{2+}$ oxidation was obtained when the chloroplast suspensions were illuminated before addition of $Mn^{2+}$ and then incubated in the dark.

*Comparison of spectrophotometric and manometric methods of estimation of the manganese oxidation product (manganipyrophosphate)*

The amount of manganipyrophosphate formed when chloroplast preparations are illuminated in the presence of added $Mn^{2+}$ was often sufficient to impart a pink colour to the supernatant solution. Under these conditions, provided that sufficient of the supernatant solution was used, it was possible to estimate the manganipyrophosphate by the manometric method. The results obtained by this method generally agreed well with those obtained by the more sensitive spectrophotometric method (Table 2).

Table 2. *Spectrophotometric and manometric estimation of manganipyrophosphate formed by illuminated chloroplast preparations*

Reaction mixtures consisting of washed tobacco or spinach chloroplast preparations (0·2–2 ml.), 1·8 ml. 0·02 M-$MnSO_4$, 3 ml. 0·1 M pyrophosphate, pH 7, in a total volume of 6 ml. were illuminated for 18 hr. at 100 ft.c. Control reaction mixtures were incubated in darkness for the same period. The reaction mixtures were then diluted to 8 ml., centrifuged, and the manganipyrophosphate present in the supernatants was estimated by the spectrophotometric and manometric methods.

| Expt. no. | Plant | Volume of chloroplast preparations (ml.) | Chlorophyll present ($\mu$g.) | Total $Mn^{3+}$ formed ($\mu$g.) Spectrophotometric method | Manometric method |
|---|---|---|---|---|---|
| 1 | Spinach | 2 | 440 | 560 | 560 |
| 2 | Spinach | 0·2 | 290 | 216 | 230 |
| | Spinach | 0·5 | 740 | 430 | 460 |
| | Spinach | 1 | 1470 | 610 | 610 |
| 3 | Spinach | 1 | 1830 | 790 | 760 |
| | Spinach | 1 | 1830 | 780 | 860 |
| | Spinach | 1 | 1830 | 770 | 780 |
| 4 | Tobacco | 2 | 540 | 410 | 430 |
| | Tobacco | 2 | 540 | 400 | 380 |
| 5 | Tobacco | 2 | 580 | 420 | — |
| | Tobacco | 2* | 580 | 18 | — |

* Chloroplast suspensions previously heated for 10 min. at 100°.

*Reduction of manganipyrophosphate by chloroplast preparations*

The chloroplast preparations were found to reduce manganipyrophosphate. This was clearly shown in reaction mixtures incubated in darkness (Table 3). With chloroplasts in the light the fall in manganipyrophosphate concentration was comparatively small and was usually observed clearly only in the first 2 hr. of the incubation. The apparent oxidation measured in this work is, therefore, the resultant of both oxidation and reduction. If the amount of this reduction in light is no greater than that in darkness it is insufficient to affect the results greatly.

chloroplast material was used within 48 hr. of preparation.

*Conditions of illumination.* The reaction mixtures (6 ml.) in 50 ml. beakers had a depth of about 5 mm. and were only occasionally shaken. Under these conditions the intensity of illumination of the bottom of the reaction mixture would be much less than that at the surface, particularly when large amounts of chloroplast preparation were present. Accordingly, the effect of continuous stirring and of varying the depth of the reaction mixtures was investigated. When the reaction mixtures were stirred by bubbling with wet air, with the same intensity of incident light (100 ft.c.), the rate of $Mn^{2+}$ oxidation increased by about 25 %. When the average depth of the reaction mixtures was reduced to 0·4 mm. by using Petri dishes as reaction vessels the rate of $Mn^{2+}$ oxidation was about 40 % higher than in unstirred reaction mixtures in beakers. When open Petri dishes were used the rate of $Mn^{2+}$ oxidation was also higher than with the unstirred reaction mixtures in beakers, but the results were erratic, possibly owing to the rapid evaporation.

Table 3. *Reduction of manganipyrophosphate by chloroplast preparations*

Complete reaction mixtures consisting of 2 ml. washed tobacco chloroplast preparation (560 $\mu$g. chlorophyll/ml.) and 130 $\mu$g. $Mn^{2+}$ in 0·05 M pyrophosphate, pH 7, were incubated for periods of 2 and 18 hr. in light (100 ft.c.) and darkness. Control reaction mixtures consisting of chloroplasts without added $Mn^{2+}$ and of manganipyrophosphate alone were incubated under the same conditions. The manganipyrophosphate remaining at the end of the incubation periods was estimated spectrophotometrically.

| Components present | Conditions of incubation | Total $Mn^{2+}$ finally present ($\mu$g.) |
|---|---|---|
| Manganipyrophosphate | 2 hr. dark | 132 |
| Manganipyrophosphate | 2 hr. light | 130 |
| Manganipyrophosphate | 18 hr. dark | 132 |
| Manganipyrophosphate | 18 hr. light | 126 |
| Chloroplasts + manganipyrophosphate | 2 hr. dark | 98 |
| Chloroplasts + manganipyrophosphate | 2 hr. light | 112 |
| Chloroplasts + manganipyrophosphate | 18 hr. dark | 84 |
| Chloroplasts + manganipyrophosphate | 18 hr. light | 124 |

*Effect of various factors on the oxidation of $Mn^{2+}$ by illuminated chloroplasts*

*Type and age of chloroplast preparation.* It is known that the inclusion of sucrose in the extraction medium helps to preserve the morphological integrity of chloroplasts; phosphate–sucrose is usually preferred to sucrose alone in the isolation of intracellular structures. The manganese-oxidizing activities of chloroplast preparations made with 0·5 M sucrose solution containing 0·04 M phosphate at pH 7 were therefore compared with those of chloroplast preparations made with phosphate alone. The rate of manganese oxidation was less with the chloroplast preparations made with phosphate–sucrose. Sucrose (0·5 M) added to the reaction mixtures had little effect on the rate of manganese oxidation.

The effect of the age of the chloroplast preparation, stored at 0°, on the manganese-oxidizing activity was very variable. With some preparations the activity persisted almost unchanged for up to 3 days; with others decreases of up to 50 % were observed in this period. For most of the work the

*Period of illumination.* The relation between the period of illumination and manganipyrophosphate formation was found to be linear during the early stages of the reaction. In most experiments the rate was approximately linear for at least 1 hr., but then decreased with time until a stage was reached (after 6–24 hr.) when further prolonged illumination produced little or no increase in the amount of manganipyrophosphate present. The rate of manganipyrophosphate formation, the duration of the linear rate, and the total amount of manganipyrophosphate formed, varied widely with different chloroplast preparations even when the amounts of these used were such as contained equal amounts of chlorophyll. These experiments were carried out by

using reaction mixtures similar to those of Table 2. The manganipyrophosphate formed was estimated spectrophotometrically by measuring the difference in absorption at 258 m$\mu$. between the reaction mixtures in light and dark.

*Light intensity.* The light intensity was varied over the range 50–200 ft.c. by placing the beakers or Petri dishes containing the reaction mixtures at suitable distances from the source of light. The differences in temperature of the reaction mixtures were negligible under the conditions used. The experiments were of short duration (0·5–1 hr.), so that the measurements were made during the period when the rate of formation of manganipyrophosphate was rapid. Results obtained with three different chloroplast preparations are shown in Fig. 3. Although the amount of manganipyrophosphate formed varies with the chloroplast preparation used, all three preparations showed the same general trend of increase in activity with increase in light intensity. In other experiments a decrease was sometimes observed at an intensity of 200 ft.c. with the reaction mixtures in Petri dishes.

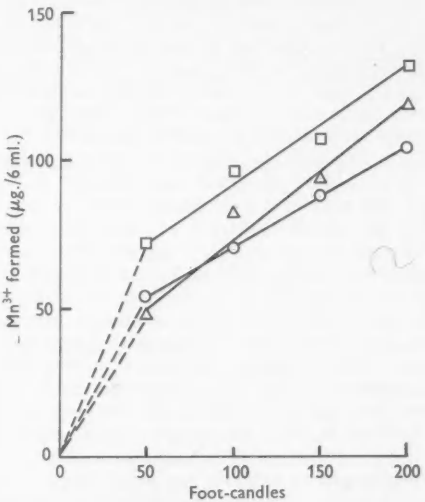


Fig. 3. Effect of light intensity on the oxidation of $Mn^{2+}$ by two different tobacco chloroplast preparations illuminated for 1 hr. in beakers △, ○; a broad-bean chloroplast preparation illuminated for 30 min. in Petri dishes, □. Reaction mixtures were similar to those of Table 2 except that 0·9 ml. 0·01 M-$MnSO_4$ was used.

*Concentration of chloroplast preparation.* It is clear from the results of Table 2 (Expt. 2) that the manganipyrophosphate formation increases with increase of chloroplast preparation. The effect of this factor on the manganipyrophosphate formation was also studied in Petri dishes by using a short reaction period (30 min.). The results (Fig. 4) show that under these conditions the manganipyrophosphate formed was approximately proportional to the chlorophyll concentration up to about 100 $\mu$g. chlorophyll/ml. Further increases in chlorophyll concentration (up to 250 $\mu$g./ml.) produced progressively smaller increases in the rate of manganipyrophosphate formation.

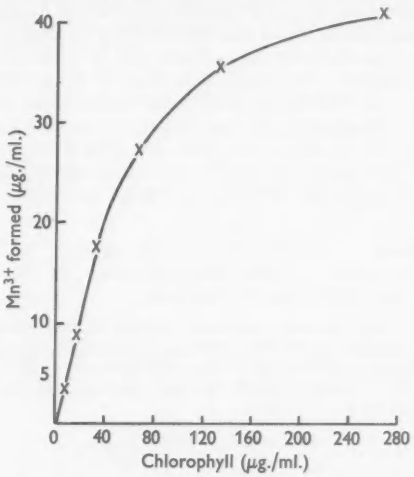


Fig. 4. Effect of varying the concentration of broad-bean chloroplast preparation on the rate of oxidation of $Mn^{2+}$. The reaction mixtures, in Petri dishes, were similar to those of Table 2 and were illuminated for 30 min. at 100 ft.c.

*Concentration of* $Mn^{2+}$. The effect of this factor on manganipyrophosphate formation was tested over a range of concentrations of $Mn^{2+}$ of 1–550 $\mu$g./ml. In these experiments, in order to keep the higher amounts of $Mn^{2+}$ in solution, the amount of buffer used was increased to 1·2 ml. M pyrophosphate. The results of one experiment are shown in Table 4. With the reaction mixtures containing the lowest concentrations of added $Mn^{2+}$ it was necessary to measure the absorption directly on the supernatants without dilution. Under these conditions the results obtained were erratic. The method finally adopted throughout these experiments was to measure the decrease in absorption at 258 m$\mu$. produced by the addition of hydrazine sulphate. This decrease in absorption was taken as a measure of the manganipyrophosphate present. At the lower concentrations of $Mn^{2+}$ the results do not represent the effect of $Mn^{2+}$ concentration on the reaction velocity

since all the added $Mn^{2+}$ was oxidized. This result is of particular interest since it shows that the reaction can take place at physiological concentrations of $Mn^{2+}$. Oxidation of added $Mn^{2+}$ was complete up to a concentration of $Mn^{2+}$ of 11 $\mu$g./ml. Further increases of $Mn^{2+}$ increased the oxidation though not in proportion to the increase in concentration of $Mn^{2+}$.

*Effect of catalase.* With reaction mixtures similar to those of Table 2 added catalase (2 mg.) caused a 20–40 % inhibition of manganese oxidation. The chloroplast suspensions contained both intact chloroplasts and chloroplast fragments of varying sizes. The fact that the inhibition by catalase was only partial may have been due to insufficient penetration of the catalase to the site of the oxidation, particularly with the intact chloroplasts and larger fragments. This was tested with two different types of chloroplast preparation. After removal of the precipitate obtained by centrifuging a tobacco leaf extract at 2000 r.p.m. (900 *g*) two fractions were obtained from the supernatant, one by spinning for 10 min. at 3200 r.p.m. (2200 *g*) and the other by spinning the 3200 r.p.m. supernatant for 10 min. at 10 000 r.p.m. (11 000 *g*). The pellets were suspended in 0·04 M orthophosphate (pH 7) and the chlorophyll contents estimated. Samples containing the same amount of chlorophyll were used in the subsequent experiments. It was found (Table 5) that the inhibiting effect of catalase was slightly more marked on the chloroplast preparations obtained by high-speed centrifuging, though in the absence of added catalase the activity of these preparations was similar to those obtained by centrifuging at lower speeds. In the reaction mixtures used the catalatic activity due to the chloroplasts present was of the order of one-hundredth of that of the added catalase.

*Effect of peroxidase.* The results given in Table 6 show that added peroxidase increases both the rate and total amount of $Mn^{2+}$ oxidation. In agreement with the known properties of peroxidase, rather prolonged heat treatment was necessary to eliminate the peroxidase effects. The amounts of added peroxidase necessary to produce the maximum increase in rate were large in comparison with the amounts of peroxidase in the chloroplast preparations. Those preparations tested showed weak peroxidatic activity in colorimetric tests with benzidine and $H_2O_2$, but the activity was usually insufficient to allow satisfactory estimations of the P.Z. by the method of Keilin & Hartree (1951). In the attempts to estimate the peroxidase activity by this method P.Z. values of 0–0·2 were obtained.

*Effect of phosphate.* When phosphate was substituted for pyrophosphate in the reaction mixtures strongly positive benzidine tests were obtained after illumination, but, on centrifuging, the supernatants had little absorption at 258 m$\mu$. These results were due to the formation of an oxidation product insoluble in phosphate. When the precipi-

Table 4. *Effect of concentration of* $Mn^{2+}$ *on the formation of manganipyrophosphate by illuminated chloroplast preparations*

Reaction mixtures consisted of 0·4 ml. washed tobacco chloroplast preparation (420 $\mu$g. chlorophyll), varying amounts of $MnSO_4$ and 1·2 ml. M pyrophosphate, pH 7, in a total volume of 6 ml. The reaction mixtures were illuminated for 3 hr. at 100 ft.c.

| $Mn^{2+}$ added ($\mu$g.) | $Mn^{3+}$ formed ($\mu$g.) |
|---|---|
| 5·5 | 6 |
| 11 | 9·5 |
| 22 | 24·5 |
| 44 | 45 |
| 66 | 62 |
| 82·5 | 64 |
| 495 | 78 |
| 1490 | 106 |
| 3300 | 167 |

Table 5. *Effect of catalase on the oxidation of* $Mn^{2+}$ *by illuminated chloroplast preparations*

The chloroplast preparations used were prepared as described in the text by low speed (LS) followed by high speed (HS) centrifuging of tobacco leaf extracts. The reaction mixtures were similar to those of Table 2 except where the catalase preparation (2 mg.) was added.

| Expt. no. | Time of illumination (hr.) | Chloroplast preparation | Chlorophyll present ($\mu$g.) | $Mn^{3+}$ formed ($\mu$g.) | | |
|---|---|---|---|---|---|---|
| | | | | No catalase added | Catalase added | Heat-treated (10 min. at 100°) catalase added |
| 1 | 1·5 | HS | 240 | 70 | — | — |
| | 1·5 | LS | 240 | 76 | — | — |
| 2 | 18 | HS | 970 | 198 | — | — |
| | 18 | LS | 970 | 190 | — | — |
| 3 | 3·5 | HS | 730 | 126 | 60 | — |
| | 3·5 | LS | 730 | 118 | 72 | — |
| 4 | 3 | HS | 590 | 78 | 40 | 64 |
| | 3 | LS | 590 | 72 | 56 | 72 |

tates obtained by centrifuging were extracted with pyrophosphate containing $MnSO_4$ the extracts obtained gave the characteristic absorption curve of manganipyrophosphate and positive tests with benzidine. Pyrophosphate containing $MnSO_4$ was used as solvent, since although $MnO_2$ is insoluble in neutral pyrophosphate it dissolves when $Mn^{2+}$ is also present to give manganipyrophosphate according to equation 1. Although $Mn^{2+}$ was added to the pyrophosphate for the routine extractions it was found that, in some cases at least, this addition was unnecessary since that part of the $Mn^{2+}$ added to the reaction mixtures which remained unoxidized during illumination of the chloroplasts was largely retained by the precipitate on centrifuging. The obtained by centrifugation from such reaction mixtures was suspended in phosphate instead of pyrophosphate for the manometric estimations, the results obtained by the method were higher than those by the spectrophotometric method. Kenten & Mann (1952*b*) demonstrated the oxidation of $Mn^{2+}$ by coupling enzyme systems producing $H_2O_2$ with peroxidase systems. Here also the oxidation product in phosphate media reacted much more slowly than manganipyrophosphate or hydrated $MnO_2$ with the hydrazine but finally gave a total $N_2$ output greater than that required by theory. The reason for these findings is not yet clear, since $MnO_2$ reacts quantitatively with hydrazine in phosphate media.

Table 6. *Effect of peroxidase on the oxidation of* $Mn^{2+}$ *by illuminated chloroplast preparations*

The reaction mixtures were similar to those of Table 2, except that 0·9 ml. 0·02 M $MnSO_4$ was used in Expt. 1.

| | Time of illumination (hr.) | Chlorophyll present ($\mu$g.) | Peroxidase added ($\mu$g.) | $Mn^{3+}$ formed ($\mu$g.) |
|---|---|---|---|---|
| Expt. 1 | | | | |
| Tobacco chloroplasts | 1 | 520 | 0 | 75 |
| | 1 | 520 | 1 | 85 |
| | 1 | 520 | 5 | 100 |
| | 1 | 520 | 50 | 125 |
| Expt. 2 | | | | |
| Spinach chloroplasts | 18 | 940 | 0 | 280 |
| | 18 | 940 | 10 | 404 |
| | 18 | 940 | 10* | 320 |
| | 18 | 940 | 10† | 304 |

* Previously heated for 15 min. at 100°. † Previously heated for 30 min. at 100°.

Table 7. *Comparison of the oxidation of* $Mn^{2+}$ *by illuminated chloroplast preparations in phosphate and pyrophosphate media*

The reaction mixtures were similar to those in Table 2 except that 0·9 ml. 0·02 M-$MnSO_4$ was used in Expt. 2, and, where indicated, phosphate buffer was used in place of pyrophosphate. The reaction mixtures were illuminated for 18 hr. at 100 ft.c.

| | | | Manganese oxidized (calculated as $\mu$g. $Mn^{3+}$) | | |
|---|---|---|---|---|---|
| | Buffer | Chlorophyll present ($\mu$g.) | Spectrophotometric method | Manometric method | Manometric method in phosphate |
| Expt. 1 | | | | | |
| Broad-bean chloroplasts | Phosphate | 1350 | 220 | 240 | — |
| | Pyrophosphate | 1350 | 250 | 240 | — |
| Expt. 2 | | | | | |
| Tobacco chloroplasts | Phosphate | 1120 | 270 | — | 390 |
| | Pyrophosphate | 1120 | 220 | 240 | — |

results of Table 7 suggest that there is little difference in the amount of manganese oxidation, whether phosphate or pyrophosphate is used to buffer the reaction mixtures. This is supported by the results both of the spectrophotometric and manometric estimations. When the manometric method was used with phosphate reaction mixtures without prior centrifuging, or when the precipitate

## DISCUSSION

The results of the present work show that $Mn^{2+}$ is oxidized by illuminated chloroplasts. This oxidation occurs at physiological concentrations of $Mn^{2+}$, though, to facilitate the estimation of the oxidation product, the experiments were generally made at relatively high concentrations of $Mn^{2+}$. $Mn^{2+}$ is

known to be present in chloroplasts, though it is apparently not concentrated therein. Thus Whatley, Ordin & Arnon (1951) found that the concentration of $Mn^{2+}$ in the chloroplasts of sugar-beet leaves was approximately equal to that expected on the basis of random distribution in the leaf. In their chloroplast preparations for each g. atom Mn, 12·8 moles chlorophyll were found, which corresponds to 4·4 $\mu$g. $Mn^{2+}$/mg. chlorophyll.

A thermolabile factor is involved. Added peroxidase increases the rate of oxidation, though the amount necessary to produce significant increases is great in comparison with that present in the chloroplast preparations used. The oxidation is partially inhibited by added catalase. As with peroxidase comparatively large amounts are necessary to produce a significant effect. A possible explanation is that these enzymes penetrate only with difficulty to the site of the oxidation. Some evidence of this has been obtained in the experiments with added catalase. The results suggest that, in part at least, the oxidation is brought about by peroxidase systems. Mehler (1951*a*, *b*) and Mehler & Brown (1952) attribute the formation of hydrogen peroxide by illuminated chloroplast preparations to the action of oxygen as a Hill reagent. It is not yet clear how far this reaction takes place *in vivo*. Mehler (1951*a*) points out that oxygen may act as a Hill reagent only under abnormal conditions, but does consider the possibility that photo-oxidations of green plants may be catalysed by peroxidases after reduction of oxygen in the Hill reaction. The results of the present work suggest the possibility that manganese oxidation occurs in chloroplasts *in vivo* and that a manganese oxidation–reduction cycle may play a part in the reactions of photosynthesis. Work in progress with pea plants (*Pisum sativum* L.) in water culture has shown that manganese oxidation can take place in plants *in vivo*. At high manganese concentrations deposition of manganese higher oxides can be demonstrated mainly in the stems, but to a lesser extent in the leaves. In view of previous work on manganese oxidation by plant extracts and peroxidase systems (Kenten & Mann, 1949, 1950, 1952*b*) it is presumed that such oxidation is due to peroxidase systems. It may be that part of the oxidation observed in the leaf is due to hydrogen peroxide produced by the action of light on the chloroplasts.*

* *Note added in proof*. Since this work was submitted for publication Andreae (1955) described a photo-induced oxidation of $Mn^{2+}$ by the use of riboflavin as light-sensitizer. The mechanism postulated could provide an explanation of the results of the present work. This possibility is under investigation.

## SUMMARY

1. Chloroplast preparations oxidize $Mn^{2+}$ in light but not, or to a much less extent, in darkness.

2. In pyrophosphate the oxidation product accumulated as a soluble manganipyrophosphate. In phosphate an insoluble oxidation product was formed which like $MnO_2$ on treatment with pyrophosphate containing $Mn^{2+}$ gave a soluble manganipyrophosphate.

3. The oxidation products oxidize hydrazine and were estimated manometrically by this reaction or spectrophotometrically as manganipyrophosphate. A sensitive spectrophotometric method of estimating manganipyrophosphate has been described.

4. The effect of variation of light intensity, period of illumination, concentration of chloroplast material and of $Mn^{2+}$ was studied.

5. Catalase partially inhibits the oxidation. Peroxidase increases the rate of oxidation.

6. It is suggested that the oxidation is due, at least in part, to peroxidase systems and that a manganese oxidation–reduction cycle may play a part in the reactions of photosynthesis.

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# A Preliminary Investigation of the Products of the Metabolism of Testosterone by Rat Liver *in vitro*

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A comprehensive summary of studies of the metabolism of androgens by animal tissues *in vitro* has been made by Samuels & West (1952). One of the points to which they draw attention, and which it is desired to emphasize here, is that, in order to permit the application of the orthodox methods of chemical manipulation, many workers choose grossly unphysiological ratios of substrate to tissue and work with suspensions or water-soluble derivatives rather than with true aqueous solutions of the steroids. This criticism is still applicable to much current work.

Thus the work of Kochakian and his associates (Clark & Kochakian, 1947; Clark, Kochakian & Lobotsky, 1947; Kochakian & Nall, 1953; Kochakian & Stidworthy, 1954) which indicates that dehydrogenases of liver or kidney act on the 3- and 17-positions and on the ethylenic linkage of testosterone, androst-4-ene-3:17-dione or 17α-hydroxyandrost-4-ene-3-one (the so-called '*cis*'-testosterone) was done entirely with suspensions of the substrates in serum–phosphate (1:1) buffer mixtures. The recently reported enzymic conversion of testosterone into androst-4-ene-3:17-dione by human serum in the presence of added diphosphopyridine nucleotide (DPN) (Richterich-van Baerle, Wotiz & Lemon, 1954) must be taken into consideration in evaluating these results. Wotiz and his associates (Wotiz & Lemon, 1954; Wotiz, Lemon & Voulgaropoulos, 1954) add the substrate to the incubation medium in solution in propylene glycol: using paper chromatography they have been able to demonstrate enzymic transformations of testosterone by a variety of tissues of which liver was the least active, contrary to the findings of Samuels, McCauley & Sellers (1947).

The development of methods for determining microgram quantities of testosterone and metabolites as $\Delta^4$-3-ketones and as 17-ketones in purified incubation extracts, and the recognition of the importance of cofactor activity (DPN in the presence of nicotinamide, citrate) are due to Samuels and his school, who obtained results which are in many points at variance with those of other workers. The position of the intermediates in their metabolic scheme must remain uncertain, until individual metabolites have been characterized. In the papers cited above, Kochakian and his school report that androst-4-ene-3:17-dione accumulates. Samuels and his school find that, while the conversion of testosterone into androst-4-ene-3:17-dione can be brought about by a purified preparation of a DPN-linked liver dehydrogenase, this steroid does not accumulate if the hormone is incubated with liver mince.

The present paper reports a new attack on the problem. A number of micro procedures were applied, including tracer studies with [4-$^{14}$C]testosterone, which was synthesized for the purpose of these experiments; conditions were used which favour 17-ketosteroid formation and ensure true aqueous solution of the substrate in the incubation medium.

## EXPERIMENTAL

Melting points were observed on a microscope hot stage and are corrected.

### *Materials*

Nicotinamide (Hopkin and Williams) was used without purification. DPN of 90% purity was kindly provided by Dr G. E. Glock (Courtauld Institute); material of 80% purity, purchased from C. F. Böhringer and Söhne, G.m.b.H., Mannheim, was used for the incubation of [4-$^{14}$C]testosterone. DPN was measured by enzymic assay with yeast alcohol dehydrogenase (Horecker & Kornberg, 1948; Racker, 1950).

Testosterone was purified by two recrystallizations from acid-free ethyl acetate, followed by adsorption on and elution from a column of 20 times its weight of neutralized alumina (Peter Spence and Co., grade H) prepared in benzene (A.R.). Pooled fractions of the benzene eluate yielded a product of m.p. 156·5–157°.

All solvents were redistilled, unless stated otherwise. Acetone was dried over $CaCl_2$ and distilled from anhydrous $K_2CO_3$. $CHCl_3$ was dried over $CaCl_2$ and freshly distilled before use. For spectrographic and colorimetric purposes, the 95% ethanol used was 'Spiritus vini rectificatus' (James Burrough Ltd.). In 17-ketosteroid and thiosemicarbazone determinations, 95% ethanol consistently gave lower blank values than the absolute ethanol available in this laboratory.

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In adsorption chromatography of incubation extracts alumina (Savory and Moore; activity grade II–III, Brockmann & Schodder, 1941) was used throughout. In partition chromatography Hyflo-Supercel (Johns–Manville Co.) and dimethyldichlorosilane-treated Hyflo-Supercel were the supporting phases. The Hyflo-Supercel was treated with 10N-HCl for 24 hr. and washed with water till free from acid. The lightest particles were removed by decantation.

2:4-Dinitrophenylhydrazine was used without further purification. Thiosemicarbazide was decolorized by treatment with animal charcoal in boiling water and recrystallized to give white needles, m.p. 185–186° (decomp.).

### *Methods employed for the separation, identification and determination of* $C_{19}$*-ketosteroids*

Unless stated otherwise, spectrophotometric measurements were made in a 1·0 cm. cell with either a Hilger Uvispek or a Unicam SP. 500 spectrophotometer. The infrared spectra were taken in $CS_2$ solution by means of a Perkin–Elmer double-beam recording infrared spectrometer (Model 21), using a microcell (3 mm. thickness).

The properties of testosterone, androst-4-ene-3:17-dione and their derivatives summarized in Table 1 were found to be useful for identification purposes.

*αβ-Unsaturated ketonic structure.* The amount of αβ-unsaturated ketone in a fixed volume of ethanol was determined by comparing the intensity of the ultraviolet absorption band at 240 mμ., against a solution of a suitable blank extract, with standard solutions of testosterone and androst-4-ene-3:17-dione. Optical densities were measured at 230, 240, 250 and 300 mμ.

*Separation and identification of ketosteroids as 2:4-dinitrophenylhydrazones.* Testosterone 2:4-dinitrophenylhydrazone and androst-4-ene-3:17-dione bis-2:4-dinitrophenylhydrazone were prepared as reference compounds (Table 1). Mixtures of the former (0·1–1·0 μmole) and the latter (0·1–0·5 μmole) could be separated quantitatively by adsorption chromatography on alumina (3 g.) prepared in *n*-hexane–benzene (1:1) (Tables 1 and 2).

Microgram quantities of ketosteroids were converted into their 2:4-dinitrophenylhydrazones by the procedure of Reich, Sanfilippo & Crane (1952); excess of reagent was destroyed by treatment with hot Benedict's solution as there described. Treatment with pyruvic acid was suggested

Table 1. *Properties of testosterone, androst-4-ene-3:17-dione and their derivatives*

| Free steroids | Testosterone | Androst-4-ene-3:17-dione |
|---|---|---|
| Elution from alumina (micro procedure) | Eluted with 0·1–0·2% ethanol in benzene | Eluted with benzene |
| Elution from Hyflo-Supercel (ethylene glycol–*cyclo*hexane) (for details see text) | Eluted with 55–72 ml. | Eluted with 8–16 ml. |
| Infrared maxima ($CS_2$) (ketone region) | 1675 cm.$^{-1}$ (sharp peak, $\Delta^4$-3-ketone) | 1675, 1740 cm.$^{-1}$ (sharp peaks, $\Delta^4$-3-ketone and 17-ketone respectively) |
| Alkaline *m*-dinitrobenzene (solution) | $\lambda_{max.}^{EtOH}$: not determined | $\lambda_{max.}^{EtOH}$: 520 mμ. |
| Alkaline *m*-dinitrobenzene (paper) | Blue (fading rapidly) | Violet (stable colour) |
| 2:4-Dinitrophenylhydrazones | | Bis derivative |
| Melting points | 206–208° | No change below 280° |
| Colour | Light red (cryst. from EtOH) | Deep red (cryst. from $CHCl_3$/EtOH) |
| Previously prepared by | Johnston (1947) | — |
| $\lambda_{max.}$ ($CHCl_3$) | 390 mμ. | 380 mμ. |
| $\epsilon_{max.}$ ($CHCl_3$) | $2{\cdot}95 \times 10^4$ | $5{\cdot}05 \times 10^4$ |
| Found | N, 11·8% | C, 57·5; H, 5·1; N, 17·0% |
| Calc. | N, 11·95% ($C_{25}H_{32}O_5N_4$) | C, 57·6; H, 5·3; N, 17·3% ($C_{31}H_{34}O_8N_8$) |
| Adsorption chromatography on alumina | Eluted with benzene–$CHCl_3$ (3:1) (sharp, light red band) | Eluted with benzene (yellow zone) |
| Thiosemicarbazones (only the testosterone derivative was prepared in milligram quantity) | | |
| Melting points | 152° (decomp.) | — |
| Colour | Very light yellow | — |
| Previously prepared by Ercoli, Koller & de Ruggieri (1951) | | |
| $\lambda_{max.}$; $\epsilon_{max.}$ (MeOH) | 247·5 mμ.; $1{\cdot}11 \times 10^4$ | 270 mμ.; $2{\cdot}34 \times 10^4$* |
| $\lambda_{min.}$; $\epsilon_{min.}$ (MeOH) | 265 mμ.; $7{\cdot}84 \times 10^3$ | — |
| $\lambda_{max.}$; $\epsilon_{max.}$ (MeOH) | 300–302 mμ.; $3{\cdot}34 \times 10^4$ | 300–302 mμ.; $3{\cdot}48 \times 10^4$† |
| Elementary analysis | Results not reproducible | — |

* (Based on log ($I_0/I$) androst-4-ene-3:17-dione bis TSC – log ($I_0/I$) testosterone TSC). MeOH 270 mμ. MeOH 270 mμ.

† (Based on log ($I_0/I$) androst-4-ene-3:17-dione bis TSC – log ($I_0/I$) androsterone TSC). MeOH 300–302 mμ. MeOH 300 mμ.

Androsterone TSC; $\lambda_{max.}$ 270 mμ.; $\epsilon_{max.}$ (MeOH): $2{\cdot}32 \times 10^4$.

Table 2. *Separation of the 2:4-dinitrophenylhydrazones of testosterone and androst-4-ene-3:17-dione*

After elution from alumina the derivatives were dissolved in $CHCl_3$ (10 ml.). Three results for each separation.

| | | Benzene–$CHCl_3$ (3:1) | | Benzene eluate | |
|---|---|---|---|---|---|
| Steroid | $\mu$moles | $E^{CHCl_3}_{390\,m\mu}$ | Recovery | $E^{CHCl_3}_{380\,m\mu}$ | Recovery (%) |
| Testosterone 2:4-dinitrophenylhydrazone | 0·2 | 0·565 | 96±0·9 | — | — |
| Androstenedione bis-2:4-dinitrophenylhydrazone (authentic specimens) | 0·1 | — | — | 0·495 | 98±0·9 |
| Testosterone* | 0·2 | 0·555 | 94±2·3 | — | — |
| Androstenedione* | 0·1 | — | — | 0·490 | 97±2·5 |
| Control*† | 0·2 | 0·530 | 90±3·3 | — | — |

* 2:4-Dinitrophenylhydrazones prepared by micro procedure, see text.
† Recovery of testosterone from boiled medium containing inactivated tissue.

(Reich, Crane & Sanfilippo, 1953) as an alternative method, but proved unsatisfactory here, even when freshly distilled acid or purified sodium pyruvate was used, owing to the persistence of neutral ketonic impurities (derivatives, $\lambda^{CHCl_3}_{max.}$ 360 m$\mu$.; eluted from alumina with benzene).

*Determination and identification of ketosteroids as their thiosemicarbazones.* Testosterone thiosemicarbazone was prepared as a reference compound (Table 1). For the preparation of the thiosemicarbazones from microgram quantities of ketosteroids, the reagent mixture of Bush (1953) was used (5 mg. recrystallized thiosemicarbazide/ml. 5% acetic acid in methanol). Methanol was distilled from 2:4-dinitrophenylhydrazine, acetic acid from $CrO_3$ and then from 2:4-dinitrophenylhydrazine. The extract containing the ketosteroid (5–50 $\mu$g.) in 95% ethanol (0·1 ml.) and the reagent (0·1 ml.) were heated for 20 min. at 95° in loosely stoppered tubes. $CHCl_3$ (15 ml.) and 0·1 N-HCl (5 ml.) were then added immediately, the stoppered tube was shaken vigorously and the HCl layer removed with a pipette. The $CHCl_3$ solution of the thiosemicarbazone was further washed with 0·1 N-HCl (5 ml.) and with water (2 × 5 ml.). It was then evaporated to dryness under reduced pressure and the residue taken up in sufficient methanol to record, at the absorption maximum, an optical density reading in the most sensitive range of the spectrophotometer. Blank determinations as a check on the 95% ethanol and the reagent were included in each series of determinations.

*17-Ketosteroid determinations.* The 17-ketosteroid content of incubation extracts was determined by the Callow, Callow & Emmens (1938) modification of the Zimmermann (1936) reaction. A 1% (w/v) solution of purified *m*-dinitrobenzene in absolute ethanol was used. Optical densities at 440, 500, 520, 540 and 600 m$\mu$. were measured (Unicam SP. 600 spectrophotometer) against a reagent blank. The 17-ketosteroid content of a standard solution was measured with each series of unknowns. The optical density of the reagent blank at 470 m$\mu$. is a measure of the concentration of interfering impurities in the reagents.

*Separation of $C_{19}$-steroids by adsorption chromatography on alumina.* The Dingemanse, Huis in't Veld & De Laat (1946) separation of urinary 17-ketosteroids suggested itself as a means of estimating the polarity range of the transformation products produced by incubating testosterone with liver tissue. The micro procedure described by Edwards, Kellie & Wade (1953) was found to be suitable for this purpose. With benzene, 0·1–0·3% ethanol in benzene, and 0·5–1·0% ethanol in benzene as eluents, known urinary $C_{19}$-ketosteroids fell into three groups. Benzene eluted the diketones 5$\alpha$- and 5$\beta$-androstane-3:17-diones and androst-4-ene-3:17-dione; androsterone, testosterone and 3$\alpha$-hydroxy-5$\beta$-androstan-17-one were in the second group; the third included alcoholic 11-oxygenated 17-ketosteroids. A micro procedure for the determination of 17-ketosteroids (Edwards *et al.* 1953) and infrared spectroscopy were employed to analyse the chromatogram fractions.

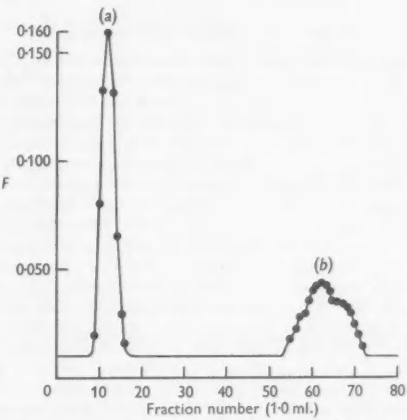


Fig. 1. Resolution of a mixture of androst-4-ene-3:17-dione (0·4 $\mu$mole) (peak *a*) and testosterone (0·4 $\mu$mole) (peak *b*). $F = 2 \log (I_0/I)_{240\,m\mu.} - [\log (I_0/I)_{230\,m\mu.} + \log (I_0/I)_{250\,m\mu.}$ measured in 95% ethanol (5 ml.). Column 10 × 1·0 cm. diam. Hold-up, 5 ml. Standard solutions of the two steroids gave $F = 0·672$ and 0·632 respectively.

*Separation of $C_{19}$-ketosteroids by partition chromatography.* Hyflo-Supercel columns (10 × 1·0 cm. diam.) were packed in the manner described by Butt, Morris, Morris & Williams (1951) and Taylor (1954). The system ethylene glycol (stationary phase; 0·5 ml./g. supporting phase) and *cyclo*hexane (mobile phase) was effective in separating androst-4-ene-3:17-dione from testosterone (Fig. 1). The flow rate was adjusted to 5 ml./hr. by means of a head of mobile phase; fractions (1 ml.) were collected with a siphon-operated fraction cutter. The mixture (0·4 $\mu$mole of each

component) dissolved in equilibrated mobile phase (0·5 ml.) was added and the tube which had contained the extract and the walls of the column rinsed with another 3 × 0·25 ml. The solvent was removed in a stream of $N_2$ and the steroid content of the fractions determined by the methods described. The proportions recovered from the column (single determination) were: androst-4-ene-3:17-dione, 95%; testosterone 91%.

*Separation of $C_{19}$-ketosteroids by paper chromatography.* The paper-chromatography system of Savard (1953) was adopted with minor modifications. Light petroleum (b.p. 100–120°) was the moving phase, and methanol–propylene glycol (1:1, v/v) the stationary phase. Whatman no. 1 filter paper was used without preliminary solvent extraction. A mixture of androst-4-ene-3:17-dione (7·5–15 μg.) and testosterone (2·5–5 μg.) could be separated and identified after running the moving phase for 5 hr.

### *Incubation of testosterone with rat-liver tissue*

*Incubation conditions.* Male white rats on the stock diet (no. 86, North Eastern Agricultural Cooperative Society, Aberdeen), average weight 170 g. and about 6 weeks old, were killed by decapitation followed by exsanguination. The livers were removed as rapidly as possible and covered with isotonic ice-cold saline till minced. For comparative purposes the preparation of the liver mince and the incubation procedure were those described by Samuels *et al.* (1947). The medium consisted of a solution (25 ml.) of DPN (6 μmoles) and 0·04 M nicotinamide in 0·04 M phosphate buffer ($KH_2PO_4$–$Na_2HPO_4$; pH 7·8). Samples (1 ml.) of an ethanolic solution of testosterone (1 μmole/ml.) were pipetted into 150 ml. conical stoppered flasks. The solvent was removed in a stream of air. Flasks containing no testosterone (blanks) were also included in the series. The residues were then dissolved in ethanol (0·2 ml.) and freshly prepared mince (500 ± 5 mg.) rinsed into the incubation flask with the medium. The flasks were then filled with $O_2$, attached to a rotating drum in a water tank kept at 37·5 ± 0·1° and incubated for 1 hr. The flasks were removed, octan-2-ol (0·2 ml.) was added to each and the contents were boiled for 5 min. to stop enzyme action; the flasks were then allowed to cool to room temperature. Controls were treated similarly, immediately after addition of the mince and before incubation, to obtain recovery data. Ether (20 ml.) was then added, the stoppers were rinsed with 95% ethanol (2 ml.) and the flasks stored in the refrigerator overnight.

### *Extraction and examination of incubation products*

*Procedure* 1. The inactivated tissue suspensions were transferred to heavy-walled centrifuge tubes (100 ml.). Extraction was effected by vigorous agitation with ether (5 × 20 ml.) followed by centrifuging at low speed and separation of the ether layers. These were combined and the solvent was evaporated. The crude extracts were purified by chromatography on alumina of the *n*-hexane solutions, in which nicotinamide remained undissolved, followed by partition between *n*-hexane and 70% ethanol (Samuels *et al.* 1947). The purity of the final extracts was adequate for measuring the $\Delta^4$-3-ketosteroid and the 17-ketosteroid content against an incubation blank. Testosterone was the standard for the $\Delta^4$-3-ketone, androst-4-ene-3:17-dione for the 17-ketosteroid determinations. Values are expressed as $10^3 \times$ μmole steroid/μmole testosterone incubated/0·5 g. tissue (wet wt.)/hr.; range in brackets (3 experiments): controls, 91 ± 0·9 (90–93) ($\Delta^4$-3-ketone); incubation, 44 ± 2·9 (39–49) ($\Delta^4$-3-ketone), 54 ± 2·6 (51–58) (17-ketone). Levedahl & Samuels (1950) find 0·50–0·76 μmole 17-ketosteroid (as androsterone)/2 μmoles testosterone incubated/g. of tissue/hr.

The infrared spectrum of the purified incubation extract had three peaks at 1703, 1740 and at 1676 cm.$^{-1}$, in order of decreasing intensity. The liver blank gave no violet colour with alkaline *m*-dinitrobenzene and showed marked, weak and no absorption at 1703, 1740 and at 1676 cm.$^{-1}$ respectively. A portion of the incubation extract in benzene was chromatographed on alumina (300 mg.) prepared in benzene. The eluting solvents were benzene (28 ml.), 0·1% ethanol in benzene (34 ml.), 0·5% ethanol in benzene (28 ml.) and ethanol (10 ml.) (cf. p. 289). Of the fractions collected (2 ml.) alternate ones were tested for the presence of 17-ketosteroids (Fig. 2). Those of the remaining fractions eluted with the same solvent were combined and the infrared spectra of the $CS_2$-soluble residues taken. The benzene fractions contained material absorbing strongly at 1676 and 1742 and very weakly at 1709 cm.$^{-1}$; fractions eluted with 0·1% ethanol in benzene absorbed weakly at 1743 cm.$^{-1}$, with 0·5% ethanol in benzene strongly at 1742 cm.$^{-1}$. Portions of the control extracts were converted into the 2:4-dinitrophenylhydrazones which were eluted from alumina with benzene–$CHCl_3$ (3:1) ($\lambda_{max.}^{CHCl_3}$ 390 mμ.). Testosterone recoveries (Table 2) were in good agreement with the values obtained on the original extract by u.v. absorption measurements. Two small, pale bands were eluted from alumina with benzene, when the 2:4-dinitrophenylhydrazone of the blank extract was similarly chromatographed; the eluate with benzene–$CHCl_3$ (3:1)

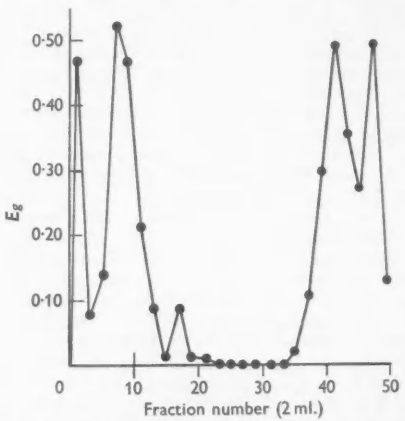


Fig. 2. Qualitative elution pattern of 17-ketosteroid metabolites. $E_g$ values are 'absorption coefficients' measured with a Spekker photoelectric absorptiometer and an Ilford Spectrum Green filter (maximum transmission at 520 mμ.). Cells of 0·5 cm. thickness and an androst-4-ene-3:17-dione standard were employed (17-ketosteroid microprocedure) (Edwards *et al.* 1953).

remained colourless. Chromatography of the 2:4-dinitrophenylhydrazones of the incubation extract gave, under identical conditions, a benzene fraction ($\lambda_{max.}^{CHCl_3}$ 380 mμ.) and another eluted with benzene–$CHCl_3$ (3:1) ($\lambda_{max.}^{CHCl_3}$ 375 mμ.), of approximately equal colour intensities.

*Procedure* 2. The incubated contents of 20 flasks (5·77 mg. testosterone) were boiled and separately extracted with ether as described above. The residues were triturated with *n*-hexane (20 ml.), the nicotinamide suspensions pooled and centrifuged at low speed. The sediment was resuspended in *n*-hexane (300 ml.), triturated vigorously and centrifuged. The clear *n*-hexane solutions were combined and shaken twice with 70% ethanol (500 ml.), the ethanolic solution was back-extracted with *n*-hexane (2 × 100 ml.) and water added to reduce the ethanol content to 30%. Extraction by $CHCl_3$ (3 × 200 ml.) of the milky-white emulsion, followed by evaporation under reduced pressure below 50°, yielded a yellow-brown, semi-solid oil; weight, after drying over $CaCl_2$ *in vacuo* overnight, 128·5 mg. 17-Ketosteroid determination indicated a 37·5% conversion by comparison with an androst-4-ene-3:17-dione standard. The extract dissolved in methanol (5 ml.)–acetic acid (0·38 ml.) containing Girard's reagent T (300 mg.) was left to stand overnight at room temperature and was then separated into ketonic and non-ketonic fractions in the usual way. The ketonic fraction, a pale-yellow, partly crystalline solid, weighed 16·7 mg. The coloured solution with *m*-dinitrobenzene and alkali now gave a symmetrical absorption curve between 440 and 600 mμ. with the peak at 520 mμ. [35% conversion (7·05 μmoles) based on the androst-4-ene-3:17-dione standard]. The purified extract gave rise to three peaks in the ketone region of the infrared spectrum, those at 1743 and 1708 cm.$^{-1}$ of equal intensity, the third at 1674 cm.$^{-1}$ half their value. Optical density measurements at 240 mμ. indicated the presence of 38·3% $\Delta^4$-3-ketosteroid (7·65 μmoles).

Orange-red 2:4-dinitrophenylhydrazones were precipitated from a portion (3·6%) of the purified extract in 95% ethanol. Adsorption chromatography using two different elution systems was employed in attempts to separate the crude 2:4-dinitrophenylhydrazone mixtures into their constituents (Fig. 3). In system 1 the residue dissolved in (1:1) *n*-hexane–benzene (5 ml.) was chromatographed under 90 mm. pressure Hg on a column of alumina (1·5 g.; 8·5 × 0·4 cm. diam.) prepared in that solvent mixture. Separation was effected by elution with benzene and benzene–ethanol mixtures. In system 2 the residue likewise dissolved in (1:1) *n*-hexane–benzene (5 ml.) was transferred to a column of alumina (3 g.; 5 × 1 cm. diam.) and eluted under gravity with benzene, benzene–$CHCl_3$, and $CHCl_3$–methanol mixtures. The yields of material behaving as androst-4-ene-3:17-dione isolated as the bis-2:4-dinitrophenylhydrazone were 20 and 16% respectively. A number of additional bands with more polar elution characteristics and absorption maxima at lower wavelengths were obtained; testosterone 2:4-dinitrophenylhydrazone was not found.

A portion (10·8%) of the ketonic extract dissolved in benzene containing 0·2% ethanol (1 ml.) was chromatographed under 90 mm. pressure Hg on alumina (1 g.; 0·4 cm. diam.) prepared in this solvent mixture. The extract was eluted from the column with 0·2% ethanol in benzene (64 ml.), 0·75% ethanol in benzene (36 ml.) and 20% ethanol in benzene (10 ml.). The first seven fractions were combined, the solvent removed in a stream of $N_2$, the residue was dissolved in (1:1) *n*-hexane–benzene (1 ml.) and rechromatographed on alumina (300 mg.) prepared in *n*-hexane–benzene (1:1). The 17-ketosteroid content of the fractions (2 ml.) was determined by the micro procedure of Edwards *et al.* (1953); the benzene eluates were determined against an androst-4-ene-3:17-dione standard, and

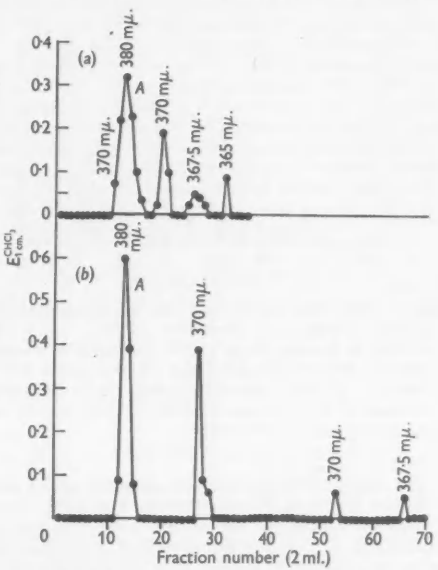


Fig. 3. Adsorption chromatography of the ketosteroid 2:4-dinitrophenylhydrazones of a portion (3·6%) of the purified incubation extract. (*a*) System 1: (1:1) *n*-hexane–benzene (20 ml.); benzene (20 ml.); 0·2% ethanol in benzene (60 ml.); 0·75% ethanol in benzene (30 ml.); 5% ethanol in benzene (10 ml.). (*b*) System 2: (1:1) *n*-hexane–benzene (20 ml.); benzene (14 ml.); (9:1) benzene–$CHCl_3$ (12 ml.); (3:1) benzene–$CHCl_3$ (8 ml.); (1:1) benzene–$CHCl_3$ (8 ml.); (3:1) $CHCl_3$–methanol (10 ml.). Values in mμ. are $\lambda_{max.}$. A, peaks ascribed to androst-4-ene-3:17-dione.

all other fractions against a dehydroepiandrosterone standard (Fig. 4). The presence of saturated diones in benzene fractions 4–7 and of androst-4-ene-3:17-dione (main benzene peak) was indicated. On this basis a 20% conversion into the unsaturated dione was calculated.

The remainder of the extract was chromatographed on alumina (3 g.) in the same manner and an identical elution pattern established by determining the 17-ketosteroid content of every third fraction. Where a peak was obtained, a number of fractions were combined and the infrared spectra of their residues taken. The second benzene fraction, while negative in the alkaline *m*-dinitrobenzene reaction, gave rise to a strong peak at 1739 cm.$^{-1}$, with lower subsidiaries at 1675 and 1775 cm.$^{-1}$, but no corresponding ester absorption between 1220 and 1250 cm.$^{-1}$. As the

main peak of the benzene eluate was approached, the band at 1675 increased in intensity compared with that at 1739 cm.$^{-1}$, until, at the centre, the two were of almost equal height. Sharp, high-intensity bands at 1739 cm.$^{-1}$ were characteristic of peak material eluted with 0·75 and 20% ethanol in benzene.

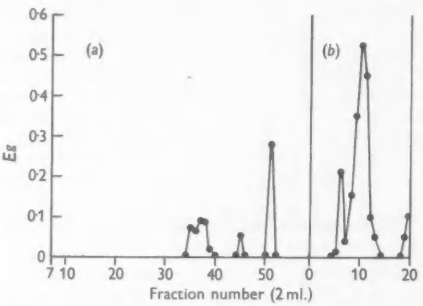


Fig. 4. Differential analysis of 17-ketosteroid metabolites, portion (10·8%) of incubation extract. (*a*) 0·2% ethanol in benzene (64 ml.); 0·75% ethanol in benzene (36 ml.); 20% ethanol in benzene (10 ml.). (*b*) The first 7 fractions of (*a*) re-chromatographed: (1:1) *n*-hexane–benzene (8 ml.); benzene (28 ml.); 0·2% ethanol in benzene (4 ml.).

The residues of the Zimmermann-positive fractions were subjected to paper chromatography. The Bush (1951) system *A* was used. Two chromatograms were run with the same fractions. I am indebted to Mr E. R. Smith for these paper-chromatography results. After the paper (Whatman no. 2) had been dried, substances were located by dipping the first paper in 2:4-dinitrophenylhydrazine reagent (Savard, 1953) and spraying the second with methanolic sodium hydroxide (Bush, 1951). The presence of androst-4-ene-3:17-dione in the fractions of the main benzene peak was indicated on comparison with an authentic specimen used as a marker. Identical $R_F$ values, orange-yellow 2:4-dinitrophenylhydrazone spots and dull, yellow-brown areas of sodium hydroxide-fluorescence (viewed under u.v. light) were obtained. A number of other substances, with lower $R_F$ values, were located in these and later fractions, reflecting the complexity of the ketonic fraction of the extract. Yellow 2:4-dinitrophenylhydrazone spots close to the solvent front pointed to the presence of saturated diones in the earliest Zimmermann-positive fractions of the benzene eluate. Later chromatogram fractions were resolved more satisfactorily with the *B* (1) system Bush (1951), but identification and proof of derivation from substrate was not possible with the quantities available. The non-ketonic fraction of the incubation extract was not investigated.

### *Incubation of* [4-$^{14}$C]*testosterone with rat-liver mince*

[4-$^{14}$C]*Testosterone*. Labelled hormone with an activity of 42 $\mu$c/m-mole was synthesized by the method of Fujimoto (1951). The crude product of the Grignard reaction of methyl iodide (2·6 mg.; 0·1 mc), diluted to 2 m-moles, and the enol lactone of 17$\beta$-acetoxy-5-oxo-4-nor-3:5-*seco*androstan-3-oic acid was hydrolysed and ring closure effected simultaneously by treatment with ethanolic NaOH. After adsorption of the crude product (210 mg.; 36% yield) on neutralized alumina (4 g.) and elution with benzene the yield of [4-$^{14}$C]testosterone (160 mg.; m.p. 152·5–155°) was 28% of the theoretical. A mixture of purified testosterone (95 mg.; m.p. 156·5–157°) and [4-$^{14}$C]testosterone (15·6 mg.) in benzene (5 ml.) was chromatographed on alumina (2 g.) prepared in benzene and eluted with the same solvent. Of the fractions (10 ml.) collected the residues with the highest melting points were dissolved in ether and combined, and the solvent was removed cautiously. The white, crystalline material (64 mg.; m.p. 155–156°) thus obtained had an activity of $20{\cdot}4 \times 10^{-3}$ $\mu$c/mg. In 95% ethanol solution it showed the characteristic u.v. absorption maximum at 240 m$\mu$. and its thiosemicarbazone showed the expected peak at 300 m$\mu$. The corresponding values of $\epsilon$, for the thiosemicarbazone the ratio ($E_{300\,m\mu.}/E_{265\,m\mu.}$), and the infrared spectrum (in a KBr window) were in good agreement with those of the testosterone standard. Paper chromatograms (cf. p. 290), in contact with the moving phase for 5, 10, and 29 hr., demonstrated the coincidence of the radioactive zone with the blue alkaline-*m*-dinitrobenzene zone and agreement of the $R_F$ value with that of a testosterone marker. In contrast with a recent report by Savard, Wotiz, Marcus & Lemon (1953), who used a tracer of higher specific activity, no residual radioactivity could be detected at or near the starting line. The radioactivity along the paper strip was measured by dragging it at a steady known speed between oppositely placed thin end-window Geiger counters.

*Radioactivity measurements*. When sufficient material was available, radioactivity measurements were made by plating the sample at infinite thickness on polythene disks (1 cm.$^2$ area) and recording the activity with an end-window Geiger counter. Radioactivity measured at infinite thickness was related to counts at infinite thinness as follows: a methanolic standard solution (10 ml.) of the diluted [4-$^{14}$C]testosterone (3·31 mg.; $20{\cdot}4 \times 10^{-3}$ $\mu$c/mg.) was prepared, portions were pipetted on to thin lens paper covering the circular plating area (2 cm.$^2$) of polythene disks and the solvent was cautiously evaporated under an infrared lamp. The radioactivity (counts/min. at infinite thinness) was simply proportional to the volume of standard solution taken and the graph relating them was practically a straight line between 0·05 and 0·5 ml.; the activities of the small portions were too low, possibly owing to pipetting errors: 0·05 ml. (61), 0·10 ml. (125), 0·20 ml. (282), 0·50 ml. (748) (no. of counts/min. at infinite thinness in parentheses). The data represent the mean of three results (range $\pm$2%). Extracts and residues to be counted *in toto* were dissolved in $CHCl_3$ or methanol (0·2 ml.), the solutions and several rinses were transferred to the disk, 0·2 ml. at a time, and the solvent was evaporated.

*Partition chromatography of* [4-$^{14}$C]*testosterone*. The labelled hormone (1·45 mg.; $20{\cdot}4 \times 10^{-3}$ $\mu$c/mg. (6180 counts/min.)) was dissolved in equilibrated *cyclo*hexane (0·5 ml.) and transferred with several rinses (4 × 0·25 ml.) to a partition chromatography column (12 × 1 cm. diam.) (p. 289). The fractions were combined as follows (counts/min. in parentheses): nos. 1–6 (0), 7–26 (200), 27–49 (45), 50–79 (5290), 80–110 (270), total eluate (5805). A paper chromatogram of the remainder (one-half) of fractions 7–26 containing radioactive contaminant was left in contact with the moving phase for 5 hr., air-dried and treated with

alkaline-DNB reagent; a brown spot, with a slightly lower $R_F$ than the value for androst-4-ene-3:17-dione, developed and faded rapidly.

*Preparation of* [4-$^{14}$C]*testosterone solution.* [4-$^{14}$C]Testosterone (10 mg.) was added to 0·04 M phosphate buffer ($KH_2PO_4$–$Na_2HPO_4$, pH 7·4), the suspension triturated vigorously at 0° in a motor-driven glass homogenizer of the Potter–Elvehjem type and centrifuged, until an optically clear supernatant solution resulted. The concentration of the solution (assuming $\epsilon^{H_2O}_{248 m\mu}$ 15 500) was 86 $\mu$g./10 ml., equivalent to 365 counts/min. Extraction of portions (10 ml.) with ether (3 × 10 ml.) and $CHCl_3$ (2 × 10 ml.) and plating of the concentrated extracts yielded 341 counts/min. (three determinations). Since aqueous solutions of testosterone kept at 0° appeared to encourage the growth of fungus after quite short periods of storage (48 hr.), the substrate solution was used immediately after preparation.

*Incubation conditions.* The livers of four male hooded rats (160 g. average weight), maintained on the Institute diet (no. 41), were minced as described by Samuels *et al.* (1947). Fresh liver mince (500 ± 5 mg.; 152 mg. dry weight) was rinsed into each of ten 150 ml. incubation flasks with a 0·04 M phosphate buffer solution (20 ml.; $KH_2PO_4$–$Na_2HPO_4$, pH 7·4) containing DPN (6 $\mu$moles), nicotinamide (0·08 M) and penicillin G (10 000 i.u.). [4-$^{14}$C]Testosterone solution (20 ml.) was then added and a stream of oxygen passed over the surface of the incubation medium for 1 min. The flasks were incubated for 1 hr. at 37·5 ± 0·1° as described (cf. p. 290) and enzyme action stopped by rapid heating to the boiling point. The contents of 10 control flasks containing buffer, DPN, nicotinamide and penicillin G, but no substrate solution, were boiled immediately after addition of the liver mince; [4-$^{14}$C]testosterone solution (20 ml.) was added and the suspensions of inactivated tissue were oxygenated and boiled for another 5 min. Fresh penicillin G (10 000 i.u.) was added, at room temperature, to each of the incubation and control flasks. They were then stored at 0° overnight and the extraction was carried out on the following morning.

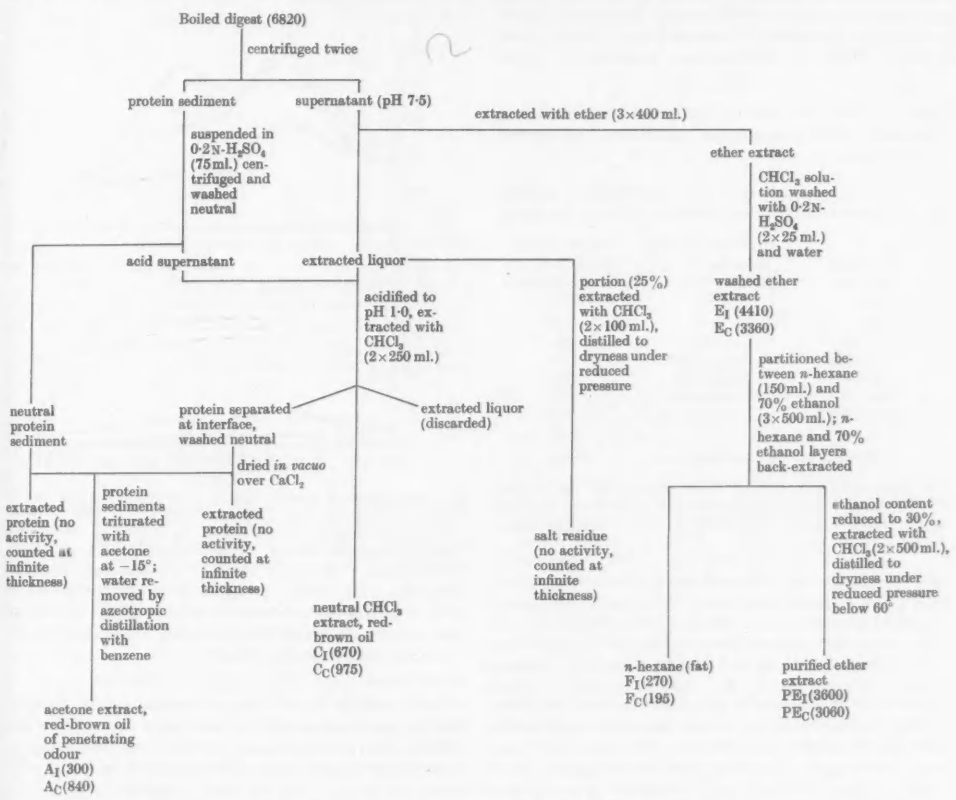
Boiled digest (6820)
centrifuged twice
protein sediment
supernatant (pH 7·5)
extracted with ether (3×400 ml.)
suspended in 0·2 N-$H_2SO_4$ (75 ml.) centrifuged and washed neutral
ether extract
$CHCl_3$ solution washed with 0·2 N-$H_2SO_4$ (2×25 ml.) and water
acid supernatant
extracted liquor
acidified to pH 1·0, extracted with $CHCl_3$ (2×250 ml.)
portion (25%) extracted with $CHCl_3$ (2×100 ml.), distilled to dryness under reduced pressure
washed ether extract $E_I$ (4410) $E_C$ (3360)
partitioned between n-hexane (150 ml.) and 70% ethanol (3×500 ml.); n-hexane and 70% ethanol layers back-extracted
neutral protein sediment
protein separated at interface, washed neutral
extracted liquor (discarded)
dried *in vacuo* over $CaCl_2$
extracted protein (no activity, counted at infinite thickness)
protein sediments triturated with acetone at −15°; water removed by azeotropic distillation with benzene
extracted protein (no activity, counted at infinite thickness)
salt residue (no activity, counted at infinite thickness)
ethanol content reduced to 30%, extracted with $CHCl_3$ (2×500 ml.), distilled to dryness under reduced pressure below 60°
neutral $CHCl_3$ extract, red-brown oil $C_I$(670) $C_C$(975)
n-hexane (fat) $F_I$(270) $F_C$(195)
purified ether extract $PE_I$(3600) $PE_C$(3060)
acetone extract, red-brown oil of penetrating odour $A_I$(300) $A_C$(840)

Fig. 5. Method of extraction and preliminary separation of products from boiled digests. I and C denote incubation and control respectively; no. of counts/min. at infinite thinness in parentheses. Total extracted: incubation (5380), control (5275).

### *Extraction and examination of the incubation products*

*Extraction and preliminary separation.* The contents of the incubation and control flasks were bulked separately, the flasks rinsed with water and the two boiled digests extracted in identical fashion. The inactivated minces were dispersed in the medium in a Townson and Mercer homogenizer run at high speed. Details of the extraction and preliminary separation of the products are given in Fig. 5. The $CHCl_3$, acetone and fat extracts (C, A and F) of the incubation digest were dissolved in $CHCl_3$ and combined; the solution was, however, lost during manipulation.

*Partition chromatography of extracts.* (i) *Purified ether extract, incubation.* The extract, dissolved in equilibrated *cyclo*hexane (1 ml.), was transferred to a Hyflo-Supercel column (12 × 1 cm. diam.). The residues of every third fraction were counted *in toto*. Sharp peaks were obtained at fractions 15 (fractions 9–12 form a shoulder) and 120. Elution with methanol stripped the stationary phase from the column; as the $CHCl_3$ solutions of these fractions were washed with water to remove ethylene glycol, some highly polar material might have been lost in the process. Peak fractions were combined, the radioactivity of portions was measured (Table 3), residues were examined by paper chromatography and the thiosemicarbazones prepared (Fig. 6). A paper chromatogram of fraction 7, portions of combined fractions 11, 13, 14, 16, 17, 19, fractions 24–100 and authentic androst-4-ene-3:17-dione (25 $\mu$g.) was scanned for radioactivity and then developed with alkaline *m*-dinitrobenzene. The marker and the samples produced violet spots of comparable colour stabilities at the same distance from the starting line and the radioactivities were concentrated in the coloured zones. Fraction 7 gave one weak violet spot (androstenedione) superimposed on a rapidly fading brown one; no Zimmermann-positive ketosteroids could be detected on the chromatogram of fractions 24–100. Portions of the pooled methylene chloride fractions containing peak radioactivity were run with two systems; the positions of the alkaline-*m*-dinitrobenzene stains on the chromatograms are shown in Figs. 7 and 8. 2α:17β-Dihydroxyandrost-4-en-3-one did not show up with alkaline DNB, but gave rise to yellow soda-fluorescence. Radioactivity was associated with the area of the strip occupied by the three spots (Fig. 7), but resolution into sharp peaks could not be obtained. The Bush chromatogram (Fig. 8) was scanned unsuccessfully after dipping in alkaline *m*-dinitrobenzene, owing to spread of the radioactivity over the entire paper strip.

Table 3. *Partition chromatography of purified ether extracts,* [4-$^{14}$C]*testosterone, incubation and control experiments*

Moving phase: fractions 1–110, *cyclo*hexane; fraction 111–128, methylene chloride; fractions 129–141, methanol.

| Fraction numbers | Incubation ($PE_I$) (counts/min. at infinite thinness) | Control ($PE_C$) (counts/min. at infinite thinness) |
|---|---|---|
| Extract | 3600 | 3060 |
| 1–6 | 0 | 0 |
| 7–26 | 985 | 165 |
| 27–49 | Negligible* | 64 |
| 50–79 | Negligible* | 2260 |
| 80–110 | Negligible* | 75 |
| 111–128 | 1210 | 200 |
| 129–141 | 170 | 48 |
| Total | 2365 | 2812 |

* Individual fractions in these groups did not exceed 8 counts/min. The fractions were not pooled and counted *in toto*, as they were required for paper chromatography.

(ii) *Ether extract, control.* The extract was subjected to partition chromatography, fractions (1 ml.) were combined in groups and their radioactivities determined (Table 3). The paper chromatogram of fractions 7–26 (one-half) (thiosemicarbazone spectrum, Fig. 6) gave rise to a rapidly fading brown spot with an $R_F$ value slightly lower than that of androst-4-ene-3:17-dione (cf. p. 293). Portions of fractions 50–79 and the methylene chloride fractions 111–128 (*in toto*) showed the characteristic blue reaction of testosterone at the same positions on the chromatogram as an authentic specimen. Fractions 27–49 and 80–110 gave blank chromatograms after exposure to the alkaline-*m*-dinitrobenzene reagent.

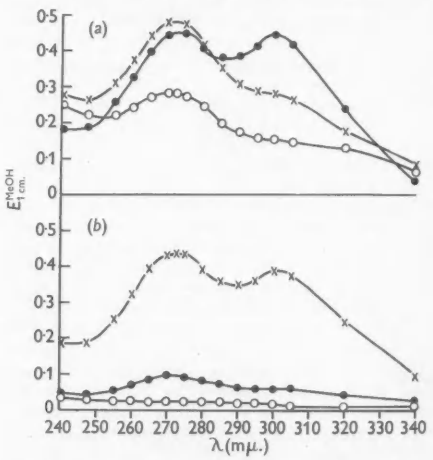


Fig. 6. Ultraviolet absorption of the thiosemicarbazones of [4-$^{14}$C]testosterone metabolites in methanol measured against reagent blanks. (*a*) ●, Standard androst-4-ene-3:17-dione (0·2 $\mu$mole; 15 ml.); ×, fractions 111–128 (6·6%; 5 ml.) $PE_I$; ○, fractions 8 and 10 (*in toto*; 15 ml.) $PE_I$; (*b*) ×, fractions 7–26 (8·4%; 5 ml.) $PE_I$; ●, fractions 7–26 (12·5%; 5 ml.) $PE_C$; ○, reagent blank (15 ml.) read against methanol.

(iii) *Combined F-, C- and A-extracts, control.* A preliminary purification of the dark-brown oily residue was effected by reversed-phase partition chromatography. Hyflo-Supercel, treated with dimethyldichlorosilane (kindly given by Dr A. T. James), was impregnated with 0·5 ml. equilibrated stationary phase ($CCl_4$)/g. supporting phase; the moving phase was equilibrated with 80% methanol. The extract in methanol (0·5 ml.; 1640 counts/min.) was stirred into a paste with the Hyflo-Supercel (2 g.) and

transferred to the top of the column (12 × 1 cm. diam.) with 80% methanol (3 × 1 ml.). A total of 1480 counts/min. were recovered from the column in six consecutive fractions (1 ml.). The first and last three fractions were separately partitioned between *n*-hexane and 70% ethanol as described and the $CHCl_3$ extracts of the ethanolic (30%) solutions subjected to paper chromatography. The characteristic, rapidly fading blue stains were found at the same distance from the starting line as the spot produced by the testosterone marker.

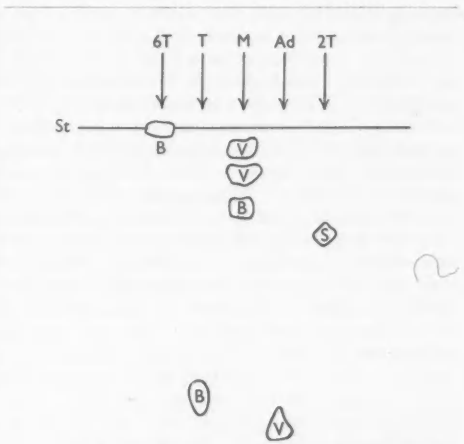


Fig. 7. Polar transformation products of [4-$^{14}$C]testosterone ($PE_I$, fractions 111–128; cf. Table 3) and reference compounds run with the modified Savard system. 6T, 6$\beta$:17$\beta$-dihydroxyandrost-4-en-3-one; T, testosterone; M, metabolites, Ad, adrenosterone; 2T, 2$\alpha$:17$\beta$-dihydroxyandrost-4-en-3-one; St, starting line; B, blue, V, violet alkaline DNB colours; S, soda-fluorescence.

## DISCUSSION

This paper reports a preliminary investigation of the products of the *in vitro* metabolism of testosterone by rat-liver mince under conditions favouring 17-ketosteroid formation. This choice provides a suitable starting-point for pathway studies, since evidence has been presented that 17-ketones are intermediates in the *in vitro* transformation of the hormone to non-ketonic substances (Samuels & West, 1952); a number of experimental methods are now available for the identification and determination of microgram quantities of ketosteroids in tissue extracts, whereas the analysis of alcoholic, non-ketonic steroids is much more difficult. Testosterone has been incubated for 1 hr. (cf. Samuels, Sweat, Levedahl, Pottner & Helmreich, 1950) in a simple phosphate buffer ($KH_2PO_4$–$Na_2HPO_4$) supplemented with DPN and nicotinamide (cf. Mann & Quastel, 1941), in the absence of heavy metals (to minimize attack of the substrate due to processes of oxidative phosphorylation, Friedkin & Lehninger, 1949), and at a pH (7·8) close to the optimum established with a purified, cell-free preparation of the DPN-linked liver dehydrogenase (Sweat, Samuels & Lumry, 1950). By employing concentrations of testosterone which are in solution in the aqueous medium, substrate:tissue ratios which approach physiological conditions have been possible. In the first isolation experiment the hormone was dissolved in a small quantity of ethanol (1 $\mu$mole testosterone/0·2 ml. ethanol/25 ml. medium). [4-$^{14}$C]Testosterone has been incubated in an entirely aqueous medium at pH 7·4.

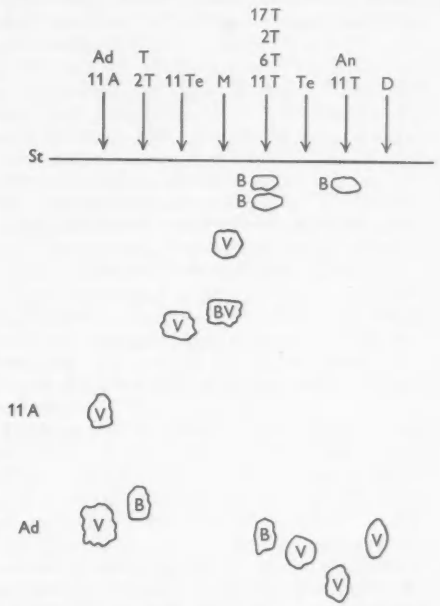


Fig. 8. Polar transformation products of [4-$^{14}$C]testosterone ($PE_I$, fractions 111–128; cf. Table 3) and reference compounds run on Whatman no. 2 paper with the system toluene–ligroin–methanol–water 10:10:9:1) for 2·5 hr. after equilibration for 3 hr. at 31°. 6T, T, M, Ad, 2T, St, B, V, as in Fig. 7. 11A, 11$\beta$-hydroxyandrost-4-ene-3:17-dione; 11Te, 3$\alpha$:11$\beta$-dihydroxy-5$\beta$-androstan-17-one; 11T, 11$\alpha$:17$\beta$-dihydroxyandrost-4-en-3-one; 17T, 17$\alpha$-hydroxyandrost-4-en-3-one *cis*-testosterone; Te, 3$\alpha$-hydroxy-5$\beta$-androstan-17-one; An, androsterone; D, dehydroepiandrosterone; BV, blue-violet alkaline DNB colour. I am indebted to Dr I. E. Bush for these paper-chromatography results.

Substantial loss of $\alpha\beta$-unsaturation and formation of material reacting as 17-ketosteroids with alkaline *m*-dinitrobenzene are confirmed (Samuels *et al.* 1947), but these structural changes cannot be explained by those authors' assumptions (Samuels & West, 1952). Only trace amounts of 3-hydroxy-17-ketones (or of other alcoholic ketosteroids of similar polarity) have been found in the incubation extracts and no unchanged testosterone has been identified. The recovery of micromolar quantities of testosterone from boiled controls has been satisfactory (91%). Enzymic transformation into androst-4-ene-3:17-dione accounts for at least 20% of the added hormone, and this metabolite is largely responsible for the remaining ultraviolet absorption peak at 240 m$\mu$. The evidence that androst-4-ene-3:17-dione is a metabolic product is as follows: the elution characteristics of the free metabolite in adsorption chromatography and column and paper partition chromatography systems and of the bis-2:4-dinitrophenylhydrazone in adsorption chromatography agree with those of an authentic specimen. With alkaline *m*-dinitrobenzene the metabolite reacts as a typical 17-ketone both in solution and on paper. The thiosemicarbazone and the 2:4-dinitrophenylhydrazone derivatives have the expected maxima in the ultraviolet and visible regions of the spectrum respectively, and the free metabolite shows the required peaks in the ketone region of the infrared spectrum. Less polar 17-ketosteroids, possibly saturated 3:17-diones, appear to be formed in trace amounts. Incubation of [4-$^{14}$C]testosterone has confirmed these findings.

Evidence is now presented that compounds more polar than testosterone are major metabolites. Incubation of the $^{14}$C-labelled compound has demonstrated that these substances are in fact derived from the substrate. On adsorption and partition chromatography they show elution characteristics similar to 11-oxygenated 3-hydroxy-17-ketosteroids and in solution give violet colours with *m*-dinitrobenzene and ethanolic alkali. Paper chromatography of the [4-$^{14}$C]testosterone incubation extract points to the presence of two polar 17-ketosteroids and a $\Delta^4$-3-ketosteroid or saturated 3-ketosteroid without carbonyl at C-17; 3$\alpha$:11$\beta$-dihydroxy-5$\beta$-androstan-17-one has a slightly higher $R_F$ value than that of the less polar of the two 17-ketones. After considerable manipulative and one accidental loss of material, radioactivity measurements accounted for 30% of the added [4-$^{14}$C]testosterone as androst-4-ene-3:17-dione (11%) and unidentified, polar ketosteroids (19%). These results show that it is not permissible to measure the 17-ketosteroid content of the incubation extract against a single standard; since the colorimetric equivalents (at 520 m$\mu$.)/mole for androst-4-ene-3:17-dione, androsterone and 3$\alpha$:11$\beta$-dihydroxy-5$\beta$-androstan-17-one are 127, 100 and 65 respectively (personal communication from Dr A. E. Kellie; cf. Wilson, 1954), a differential analysis of the extract is required.

Samuels *et al.* (1947) have observed that the addition of citrate enhances the destruction of the $\alpha\beta$-unsaturation of testosterone by liver tissue. It is not clear whether this action is specific. The mechanism of action of citrate may be explained by the coupling of the aconitase reaction and the TPN-specific oxidation of *iso*citric acid with $\Delta^4$-3-ketone reduction. A stereospecific addition of hydrogen (cf. Loewus, Westheimer & Vennesland, 1953) catalysed by two enzymes would account for the isolation of labelled androsterone and 3$\alpha$-hydroxy-5$\beta$-androstan-17-one (aetiocholanolone) in varying ratios from urine extracts after administration of labelled testosterone (Fukushima, Bradlow, Dobriner & Gallagher, 1954). As members of the citric acid cycle have been excluded from the experiments now reported, mediation of hydrogen transfer to the $\Delta^4$-3-ketone group by reduced triphosphopyridine nucleotide is limited by the concentration of endogenous substrates. As expected, enzymic oxidation of the 17$\beta$-hydroxyl group occurs readily under the conditions of the experiments now reported. Whether the reduced DPN formed by dehydrogenase action at C-17 is required and available as a cofactor for the reduction of the $\alpha\beta$-unsaturation remains to be tested by experiment.

Hydroxylation reactions are postulated to account for the formation of the polar 17-ketosteroids. $C_{19}$-Steroids with hydroxyl groups at C-11 or C-16 have been isolated from human urine (Samuels & West, 1952; Hirschmann, 1943; Lieberman, Praetz, Humphries & Dobriner, 1953); a steroid oxygenated at C-7 has also been found but may be an artifact (Fukushima, Kemp, Schneider, Stokem & Gallagher, 1954). It has generally been assumed that 11-oxygenated steroids have an adrenal origin (Dobriner & Lieberman, 1950). The mechanism of this hydroxylation reaction has been widely studied (Sweat, 1951; Hayano, Dorfman & Yamada, 1951; Kahnt & Wettstein, 1951; Brownie & Grant, 1954); the adrenal enzyme appears to be concentrated in the mitochondria and can be primed by any citric acid cycle intermediate, preferably fumarate or $\alpha$-oxoglutarate. Little information is available as to the site of 16-hydroxylation (cf. Schneider & Mason, 1948). The possibility that 16-hydroxy-17-ketosteroids may have been formed under the conditions described above is of particular interest, as further metabolism of these compounds may result in the rupture of the steroid D-ring. It is suggested that a search among the steroids of the more highly oxygenated urinary fractions for excretory end-

products of testosterone metabolism of this type may prove fruitful.

Further investigation is required to identify these polar compounds and to account for the testosterone added in terms of its major metabolites.

## SUMMARY

1. The metabolism of testosterone by rat-liver mince *in vitro* has been studied at concentrations ensuring aqueous solution of the hormone and under conditions favouring 17-ketosteroid formation.

2. Testosterone has been incubated in phosphate buffer (pH 7·8) supplemented with diphosphopyridine nucleotide and nicotinamide, but in the absence of heavy metals. At least 20% of the substrate is converted into androst-4-ene-3:17-dione. Enzymic transformation into unidentified polar ketosteroids accounts for a large fraction of the incubated hormone. Hydroxylation reactions are postulated to explain their formation. Androsterone, 3α-hydroxy-5β-androstan-17-one, and residual testosterone have not been detected in the extracts.

3. Incubation of [4-$^{14}$C]testosterone at pH 7·4 has confirmed these findings.

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# 2-Carboxypyrrole: its Preparation from and its Precursor in Mucoproteins

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Over the past six years this laboratory has been engaged in an investigation of the chemical composition and of the pattern of certain mucoproteins. Interest in this class of compounds was greatly stimulated by Burnet and co-workers when they discovered that a number of mucinous secretions of human and of animal origin inhibited haemagglutination by influenza virus, but irreversibly lost this capacity upon treatment with living influenza virus or with a soluble enzyme obtained from *Vibrio cholerae* cultures referred to as receptor-destroying enzyme (RDE) (for review see Burnet, 1952). Already the first attempts to purify the biologically active agent of the mucinous material left little doubt as to its mucoprotein character (McCrea, 1948). When a homogeneous mucoprotein inhibiting virus haemagglutinin at very low concentration ($1 \times 10^{-5}$ g./l.) became available (Tamm & Horsfall, 1952; Ada & Gottschalk, 1952), it was shown that interaction between the inhibitor and purified influenza virus B resulted in the release of a diffusible compound, thought to be an *N*-substituted *iso*glucosamine (Gottschalk, 1951). The diffusible split product gave a positive Molisch test, had reducing power (in alkaline medium) and decomposed with considerable humin formation on heating with dilute mineral acid; it coupled with *p*-dimethylaminobenzaldehyde to give a stable purple colour, even after prolonged heating with alkali, but failed to do so after treatment with mineral acid. A compound with the same properties was obtained when RDE instead of virus was used as the catalytic agent.

Though the isolation of the split product clearly demonstrated the activity of the viral enzyme and thus disposed of the claim (Bauer, 1949) that viruses are devoid of enzymes, the chemical identification was rendered difficult by the small yield. Relevant information, however, was gained by an investigation of the mucoprotein. The presence in the split product of a sugar residue and the sensitivity of the biological activity of the mucoprotein to treatment with periodic acid at low concentration (Burnet, 1948) suggested that the carbohydrate moiety of the mucoprotein contained the segment involved in the enzymic process. The urine mucoprotein was found on analysis to contain hexosamine, galactose, mannose and fucose in the molar ratio 8:6:3:1, the hexosamine fraction being a mixture of glucosamine and galactosamine (Gottschalk, 1952; Odin, 1952). The polysaccharide could be detached from the protein by alkali treatment and its molecular size was shown to be smaller than that of lysozyme (molecular weight 15000). This relatively small polysaccharide may be regarded as the prosthetic group of the urine mucoprotein, about 150 of them being attached to each molecule.

Acid hydrolysis of the prosthetic group was accompanied by the formation of considerable amounts of dark humin matter, indicating the location in the polysaccharide of the compound released from the mucoprotein by viral enzyme or RDE action. A compound with similar properties was first isolated in crystalline form from bovine submaxillary-gland mucin by Blix (1936) in a yield of 0·075–0·150% and recently termed 'sialic acid' (Blix, Svennerholm & Werner, 1952). The reducing monobasic acid of unknown structure, with the probable composition $C_{14}H_{23}O_{11}N$ (but see Discussion) was characterized by strong humin formation and liberation of carbon dioxide on heating with mineral acid, by the purple colour it gave with Ehrlich *p*-dimethylaminobenzaldehyde reagent even without alkali pretreatment (direct Ehrlich reaction) and by a violet colour when treated with Bial's orcinol reagent. Klenk & Lauenstein (1952) prepared from haemagglutinin-inhibitory mucoproteins (human urine and bovine submaxillary-gland mucoprotein) the crystalline methoxy derivative of neuraminic acid, $C_{11}H_{21}O_9N$, isolated first from gangliosides (Klenk, 1941) and exhibiting some properties again similar to those of the split product. Kuhn & Brossmer (1954) obtained the crystalline methoxy derivative of lactaminic acid, $C_{11}H_{19}O_9N$, from cow colostrum, a compound resembling neuraminic and sialic acid in some of its reactions, and Trucco & Caputto (1954) prepared from rat mammary glands a substance, neuraminlactose, consisting of neuraminic acid and lactose. Finally it was observed that the various virus haemagglutinin-inhibitory mucoproteins, and more specifically the prosthetic group and the compound released from it by viral enzyme action, gave the

direct Ehrlich reaction (Odin, 1952; Gottschalk, 1953, 1954*a*).

These findings taken together directed our attention to a pyrrole derivative as the probable acid-labile, alkali-stable structure responsible for the direct Ehrlich reaction of the biological material tested. In a preliminary note the isolation and identification of 2-carboxypyrrole from the alkali hydrolysate of bovine submaxillary-gland mucoprotein were reported (Gottschalk, 1953). We have since duplicated the results with homogeneous preparations of bovine submaxillary-gland and human-urine mucoprotein, using an improved method of purification. The 2-carboxypyrrole thus obtained in a yield of about 0·05 and 0·03 % respectively of the mucoprotein was found to be contaminated with about 1 % of a second, not yet identified, pyrrole derivative. Separation of the two compounds was achieved by chromatography.

As was expected, the same imino acid could be prepared from the product liberated from the mucoprotein by enzyme action. In this case 2-carboxypyrrole only, i.e. free from contamination by the second pyrrole derivative, was obtained in a yield of 0·03–0·05 % of the mucoprotein.

It was surprising to find that 2-carboxypyrrole is not preformed as such in the biological material, but derives from a labile precursor, probably of the 2-carboxyhydroxypyrroline type, stabilized within the natural framework by substitution of the hydroxyl group but becoming rearranged readily to 2-carboxypyrrole on release of the substituent (Gottschalk, 1954*b*).

This paper gives a detailed account of the isolation, purification and identification of 2-carboxypyrrole from two homogeneous mucoproteins and from the diffusible compound resulting from enzyme action on the mucoprotein substrate. The evidence for identification rests on the catalytic reduction of the isolated compound to 2-carboxypyrrolidine (proline) and on the identical chromatographic and spectroscopic behaviour of the compound and of authentic 2-carboxypyrrole. The paper also provides evidence for the presence in mucoproteins of a precursor which upon very mild and short alkali treatment is transformed into 2-carboxypyrrole.

Two claims about the isolation and identification of 2-carboxypyrrole from biological material have been made previously. Minagawa (1946) hydrolysed a purified preparation of yeast invertase with 25 % sulphuric acid for 5 hr. at 100° and extracted an amino acid with ethanol from the neutralized and concentrated hydrolysate. The crystalline substance, termed 'minaline', exhibited the same physical and chemical properties, including a positive ninhydrin reaction, as proline, but gave a 'special spectrogram'. The author did not compare 'minaline' with an authentic sample of 2-carboxypyrrole. The published spectrogram of 'minaline' does not resemble that of authentic 2-carboxypyrrole, and its positive reaction with ninhydrin, as well as the use of 25 % sulphuric acid in its preparation, justifies the statement that 2-carboxypyrrole was not the amino acid isolated by Minagawa. Hiyama (1949) treated bull sublingual-gland mucin with 10 % (w/v) KOH for 10 hr. at 100° and isolated by an elaborate procedure from the hydrolysate a crop of crystals in a yield of 0·08 % of the original material. By elementary analysis the compound was identified as 2-carboxypyrrole. Hiyama regarded the isolated imino acid as a degradation product by hot concentrated alkali of a completely substituted pyrrolidine having a glucosamine residue incorporated in its structure in such a manner as to share two carbon atoms with the amino sugar. It is not known whether pyrrolidines are transformed into pyrroles under the conditions applied, and Hiyama's results cannot therefore be interpreted. It would appear that the use by Hiyama of hot concentrated alkali in the preparation of 2-carboxypyrrole has obscured the realization that the pyrrole compound is not the degradation product of a stable saturated heterocyclic substance, but rather derives by rearrangement from a labile precursor of the hydroxypyrroline type. In our hands the lengthy procedure described by Hiyama is not a practical way to obtain crystalline 2-carboxypyrrole from mucoproteins.

## MATERIALS

*Preparation of bovine submaxillary-gland mucoprotein.* Bovine submaxillary glands freshly collected at the abattoirs were immediately submitted to the procedure described by Curtain & Pye (1955). Purification in this procedure is achieved by methanol fractionation at low temperature in the presence of barium ions. The final mucoprotein solution was exhaustively dialysed against water and used either as such or after freeze-drying, storage *in vacuo* and reconstitution. In electrophoresis it moved as a single peak in phosphate buffer pH 7·0 and $I = 0{\cdot}2$.

*Preparation of human-urine mucoprotein.* The procedure of Ada & Gottschalk (1952) was followed.

*Synthesis of 2-carboxypyrrole.* Pyrrolylmagnesium iodide was prepared by Oddo's (1909) method, the optimum conditions developed for the Grignard reagent by Gilman & Meyers (1923) being applied. Dry $CO_2$ was passed through the ether solution of the compound. Instead of the resulting product being decomposed with $H_2SO_4$, an excess of N-NaOH was added, the mixture heated to boiling and the $Mg(OH)_2$ formed filtered off (McCay & Schmidt, 1926). After concentration and acidification with HCl pure, white crystals of 2-carboxypyrrole were obtained in satisfactory yield, m.p. 189–190°; on a filter-paper chromatogram the material ran as a single spot in all solvents tested.

*Receptor-destroying enzyme (RDE).* This was prepared from the filtrate of *Vibrio cholerae*, as described by Ada & French (1950).

## METHODS

*Paper partition chromatography.* One-dimensional descending chromatograms were run on Whatman no. 1 paper strips (8 in. × 18½ in.) in glass jars of 10½ in. diameter and 20 in. height. Three μl. of the solutions were applied as individual spots along a line ruled 2¾ in. from the narrow edge of the strip. As markers authentic samples of known substances were run in a concentration approximating to that of the unknown, an adjustment usually requiring a preliminary run. For elution purposes the solution was applied in a 0·5 in. band along the starting line. The papers were inserted into a stainless-steel trough and held in position by two glass plates 8½ in. × 1¾ in. No provision was made for constant temp.

The solvents were butanol–acetic acid–water (4:1:5, by vol.), butanol–pyridine–water (6:4:3, by vol.), *s*-collidine saturated with water and phenol saturated with water; in the last case a few crystals of KCN were added to the stationary phase of water saturated with phenol. Commercial *n*-butanol was rectified in a fractionating column and the fraction distilling at 117–118° collected and used. Phenol was purified by steam distillation and the two phases of the distillate were separated appropriately. Commercial *s*-collidine was purified as described by Partridge (1948). Pyridine, A.R. grade, was redistilled before use.

After the run the papers were dried at room temp. in a current of air for several hours. Chromatograms run on washed filter paper for elution of compounds were dried for 24 hr. or longer to ensure complete evaporation of the solvent. Elution was carried out in a glass chamber, as described by Aminoff & Morgan (1951).

Pyrrole derivatives are easily traced with an Ehrlich reagent prepared by dissolving 100 mg. *p*-dimethylaminobenzaldehyde in a mixture of 3·0 ml. conc. HCl and 3·0 ml. ethanol and adding 18·0 ml. *n*-butanol. The isatin spray contained 0·2 g. isatin in a mixture of 4·0 ml. acetic acid and 96·0 ml. *n*-butanol (Acher, Fromageot & Jutisz, 1950).

*Spectrophotometry.* A Hilger Uvispek photoelectric spectrophotometer and 0·5 cm. silica cells were used for the ultraviolet examination. The wavelength scale of the apparatus was calibrated by means of a mercury–hydrogen arc lamp.

*Catalytic reduction of 2-carboxypyrrole.* 2-Carboxypyrrole is reduced only with difficulty to 2-carboxypyrrolidine (proline). Following mainly Putokhin (1930) the procedure was as follows: 10 mg. platinum oxide (Adams's catalyst) and a mixture of 0·5 ml. acetic acid and 0·5 ml. ethanol was placed in a 25 ml. Erlenmeyer flask and, after removal of the air and equilibration with $O_2$-free $H_2$, shaken in $H_2$ for 2 hr. at room temp. 2-Carboxypyrrole (24·0 mg.) and 1 drop of a solution containing 6·3 g. $FeCl_3$/100 ml. water were added to the reduced catalyst and shaken in $H_2$ for 45 min. at room temp. Finally 0·2 ml. ethanol containing 0·19 g. HCl/ml., 1 drop of the $FeCl_3$ solution and another 10 mg. catalyst were added and the mixture was shaken for 3 hr. in $H_2$ at room temp. Occasionally $H_2$ consumption did not proceed with the material of biological origin. In this case a preliminary treatment of the material with the catalyst in order to adsorb to its surface poisoning substances, followed by removal of the catalyst, proved useful. At the end of the procedure the contents of the flask were filtered, 5 ml. water added and the mixture was dried in a desiccator over KOH and $H_2SO_4$. On chromatographic analysis of an ethanol extract of the dried material the presence of proline was established by the blue colour of a spot reacting with isatin and coinciding in $R_F$ value and colour shade with an authentic sample of L-proline run as marker on the same strip. $R_F$ values found for proline in the solvents mentioned and in the same order were: 0·31, 0·04, 0·15, 0·86.

*Direct Ehrlich reaction.* To test for the presence in mucoproteins and proteins of a component reacting directly with Ehrlich reagent 1 vol. of reagent was added to 5 vol. of a 2% (w/v) solution of the protein and the mixture heated at 100° for 10 min. In a positive test a stable purple colour soon appears, deepening on further heating. Reagent: 500 mg. *p*-dimethylaminobenzaldehyde were dissolved in a mixture of 5·0 ml. conc. HCl and 5·0 ml. water (Werner & Odin, 1952). Tryptophan did not produce a stable purple colour under these conditions.

*Dry matter.* A portion of the mucoprotein solution was dried at 92° and weighed.

*Haemagglutinin-inhibitory activity of mucoprotein.* This was determined as described by Burnet (1948), heated PR8 influenza virus being used as indicator.

*RDE activity.* This was determined as described by Burnet & Stone (1947).

### *Preparation of 2-carboxypyrrole from mucoproteins*

(1) To 500 ml. of a 1% (approx.) (w/v) solution of the bovine submaxillary-gland mucoprotein were added 50 g. $Ba(OH)_2,8H_2O$, 10 ml. ethanol and 1·0 ml. octan-2-ol, and the mixture was refluxed in $N_2$ at 100° for 8 hr. After cooling, $CO_2$ was slowly bubbled through the hydrolysate and the precipitate formed was removed and washed with water. The pooled supernatant liquid and washings (pH 7·5) were concentrated under diminished pressure at 40° to a small volume, filtered and extracted with ether, and the ether was discarded. The aqueous phase was ice-cooled, acidified to pH 4·0 with N oxalic acid or with N-$H_2SO_4$ and the acid solution after removal of the precipitate extracted several times with ether, the pH of the aqueous phase being readjusted between extractions. The combined ether extracts were concentrated *in vacuo* and washed with water. After separation from the water phase the ether layer was thoroughly dried over anhydrous $Na_2SO_4$ and the ether evaporated *in vacuo* at low temp. The dried residue formed a snow-white, thin film covering the glass wall; on exposure to air it gradually assumed a reddish colour. On chromatographic analysis this material showed the presence of two compounds reacting directly with Ehrlich reagent, one coinciding with authentic 2-carboxypyrrole, the other being a faster-running substance of unknown structure. On repeated addition of water and drying *in vacuo* at 20° the faster-running compound, being somewhat volatile with water vapour, could be largely removed, the final product containing not more than 1% of the unknown contaminant. The average yield was of the order of 0·05% of the mucoprotein as determined spectrophotometrically, the highest yield obtained being 0·1%. This material was used for analysis by chromatography and for catalytic reduction. For spectrophotometric analysis an eluate of the spot coinciding chromatographically with authentic 2-carboxypyrrole was prepared. Earlier work was carried out with a preparation containing

about 7·5% of the contaminant; obviously for chromatographic analysis of the two compounds and for the spectroscopic analysis of their respective spots this was no disadvantage.

(2) Human-urine mucoprotein (2 g.) was treated in the manner described under (1). Average yield of 2-carboxypyrrole, 0·03%; contamination, as above (1).

*Preparation of 2-carboxypyrrole from the diffusible product split from mucoprotein by enzyme action*

(3) A solution (300 ml.) containing approximately 1% (w/v) bovine submaxillary-gland mucoprotein (virus haemagglutinin-inhibitory titre of solution, 85 000) was digested for 5 hr. at 37° (pH 7·0) with 1·0 ml. RDE solution containing $10^6$ units enzyme, when the inhibitory titre was reduced to less than 100. The mixture was then dialysed against 10 vol. of distilled water for 48 hr. at 4° and the diffusate concentrated under diminished pressure at 40° to about 20 ml. Half of the concentrate was heated in three portions with alkali (0·1 N-$Na_2CO_3$, 0·1 N-NaOH and N-NaOH final concentration respectively) at 100° for a period of time (see Results) and then treated in the same way as under (1) but with omission of the last step. The yields of 2-carboxypyrrole were about 0·03% referred to the mucoprotein. The other half of the concentrate was not treated with alkali, but was otherwise submitted to the same procedure.

(4) A 1% (w/v) solution (100 ml.) of human-urine mucoprotein (inhibitory titre, $25 \times 10^4$), saturated with toluene, was digested with 1·0 ml. RDE solution ($1 \times 10^6$ units) for 24 hr. at 37° (pH 7·0), i.e. for a much longer period than that required for reduction of the inhibitory titre to less than 100. The mixture was then dialysed against 10 vol. of distilled water for 48 hr. at 4°. Samples of the diffusate and of the non-diffusible material were taken and the intensities of their respective colour formation with Ehrlich reagent compared in a colorimeter; the result indicated a release by enzyme action of 55% of the substance reacting directly with Ehrlich reagent. The residual diffusate and the residual non-diffusible material were then processed as under (3) and (1) respectively, the conditions of alkali treatment in (3) being 0·2 N-NaOH final concentration, 100° for 40 min. The yield of 2-carboxypyrrole thus obtained from the diffusate (split product) was about 0·05%, and from the non-diffusible material (residual mucoprotein) about 0·01% of the total mucoprotein used.

## RESULTS

*Identification as 2-carboxypyrrole of the compound prepared from mucoproteins by alkali treatment*

*Colour reactions.* Both synthetic 2-carboxypyrrole and the material prepared from bovine submaxillary-gland and human-urine mucoproteins reacted instantly in the cold with Ehrlich reagent, giving very similar purple colours; with both, the ninhydrin and Molisch reactions were negative.

*Paper partition chromatography.* Chromatography of the final products from the two mucoproteins revealed a purple spot when sprayed with Ehrlich reagent. This spot coincided in colour shade and $R_F$ value with that given by authentic 2-carboxypyrrole. This acid will give a well-defined round spot only at low concentration; its application at higher concentration results in trailing. Only when a higher concentration of the final product was used was a faint faster-moving spot reacting directly with Ehrlich reagent detectable. At this stage of purification the ratio slow (2-carboxypyrrole) spot:fast spot was about 100:1, whereas in the neutralized hydrolysate before any further treatment this ratio approximated to 1:5. These ratios were obtained by assuming each compound to give the same colour yield and determining the minimum concentration necessary to give a detectable spot. Thus in the neutralized hydrolysate five times as much solution was required to detect 2-carboxypyrrole as for the contaminant (faster spot). The faster spot never trailed, and produced with Ehrlich reagent a colour very similar to that given by 2-carboxypyrrole. Spraying of the final product with aniline hydrogen phthalate and ninhydrin respectively did not result in the formation of any spots. When eluates of the two compounds reacting with Ehrlich reagent were prepared and rechromatographed separately, they ran as single spots with the same $R_F$ values as before. Table 1 summarizes the $R_F$ values in different solvents of the material obtained from submaxillary-gland mucoprotein. Nearly identical figures were found with the material prepared from urine mucoprotein. The higher $R_F$ value given previously (Gottschalk, 1953) for the run in phenol could not be reproduced.

When synthetic 2-carboxypyrrole was submitted to the same chemical procedure as the mucoprotein (method 1) it did not change its chromatographic behaviour; after recovery from the ether extract it ran as a single spot with the same $R_F$ value as before treatment. Freshly distilled, colourless

Table 1. *$R_F$ values in various solvents of synthetic 2-carboxypyrrole and of two compounds present in a purified extract from submaxillary-gland mucoprotein*

| Solvent | Synthetic 2-carboxypyrrole | Compounds of biological origin | |
|---|---|---|---|
| | | Upper spot | Lower spot |
| Butanol–acetic acid–water | 0·87 | 0·87 | 0·92 |
| Butanol–pyridine–water | 0·60 | 0·60 | 0·81 |
| *s*-Collidine | 0·58 | 0·58 | 0·71 |
| Phenol | 0·48 (trailing) | 0·48 (trailing) | 0·71 |

pyrrole did not lend itself to chromatographic investigation; owing probably to its volatility it could not be traced on paper after a run and appropriate treatment of the chromatogram. 2-Carboxypyrrole is very sensitive towards treatment with mineral acids. When the crystalline acid was heated in 0·4N-$H_2SO_4$ for 90 min. at 100° in a sealed tube, the $H_2SO_4$ removed as $BaSO_4$, the supernatant liquid concentrated *in vacuo* and run on paper, no Ehrlich-reacting spot was detectable.

*Ultraviolet absorption spectrum.* The ultraviolet absorption spectra of synthetic 2-carboxypyrrole and of the eluate of the spot obtained from submaxillary-gland mucoprotein and coinciding chromatographically with 2-carboxypyrrole are shown in Fig. 1. Between 220 and 290 mμ. the absorption curves are nearly coincident and their maxima are identical. The ultraviolet absorption spectrum of

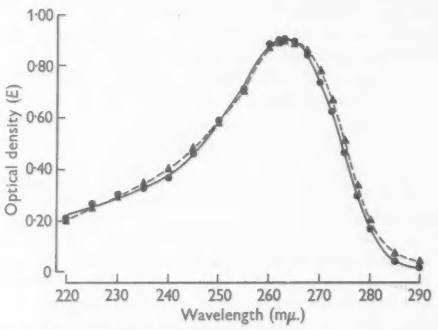



Fig. 1. Ultraviolet absorption spectra in ethanol of synthetic 2-carboxypyrrole and of the compound isolated from the alkali hydrolysate of bovine submaxillary-gland mucoprotein after removal of the contaminant. ●, Synthetic 2-carboxypyrrole; ▲, compound coinciding chromatographically with 2-carboxypyrrole.

the material obtained from urine mucoprotein was close to that of synthetic 2-carboxypyrrole, with maximum absorption at 263 mμ. In all these measurements the material was dissolved in ethanol.

*Catalytic reduction.* On catalytic reduction with Adams's catalyst (see Methods) the material isolated from submaxillary-gland mucoprotein gave rise to the formation of a substance which, in the solvents described, coincided chromatographically with authentic 2-carboxypyrrolidine (proline). No proline was detectable before reduction. When synthetic 2-carboxypyrrole in quantity equalling three times that of the contaminant present in the final product (see method 1) was submitted to catalytic reduction, the proline formed was undetectable by chromatography.

*Molecular extinction coefficient of 2-carboxypyrrole*

The ultraviolet absorption spectrum of synthetic crystalline 2-carboxypyrrole dried over $P_2O_5$ in a desiccator and dissolved in phosphate buffer (pH 7·0, $I = 0·05$) is given in Fig. 2. At 256 mμ. ($\lambda_{max.}$) $\epsilon = 11\,700$.

*Ultraviolet absorption spectrum of untreated mucoprotein*

In view of the high value of the molecular extinction coefficient of 2-carboxypyrrole and of the presence in submaxillary-gland and urine mucoprotein of the component (or components) responsible for the direct Ehrlich reaction in concentrations of 5–10% (w/w) (Odin, 1952; Klenk & Lauenstein, 1952) an attempt was made to trace

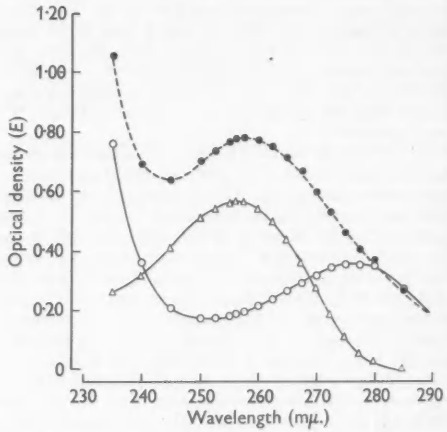



Fig. 2. Ultraviolet absorption spectra in phosphate buffer (pH 7·0, $I = 0·075$) of untreated 0·0435% (w/v) urine mucoprotein (○), 0·00115% (w/v) synthetic 2-carboxypyrrole (△), and a mixture of the two substances at these concentrations (●).

2-carboxypyrrole in the undegraded mucoprotein by its characteristic absorption maximum at 256 mμ. (in phosphate buffer). Since at low concentration the homogeneous urine mucoprotein provides a clear colourless liquid, a 0·087% (w/v) solution of this mucoprotein in phosphate buffer (pH 7·0, $I = 0·075$) was diluted with an equal volume of phosphate buffer and of a 0·0023% (w/v) 2-carboxypyrrole solution respectively and submitted to spectroscopic analysis. As may be seen from Fig. 2, no absorption peak at 256 mμ. is detectable in the untreated mucoprotein, whereas addition to the mucoprotein solution of 2-carboxypyrrole in a concentration amounting to 2·6%

(w/w) of the dry weight of the mucoprotein present resulted in a mixture exhibiting an ultraviolet absorption spectrum with an easily recognizable peak at 256 mμ.

*The split product: transformation by alkali of a precursor into 2-carboxypyrrole*

As with the untreated urine mucoprotein, spectroscopic analysis of the compound released from bovine submaxillary-gland mucoprotein by RDE at pH 7·0 and 37° failed to reveal the presence of 2-carboxypyrrole. However, after treatment of the split product with alkali under mild or more severe conditions (0·1 N-$Na_2CO_3$ for 20 min.; 0·1 N-NaOH for 45 min.; N-NaOH for 60 min.; 100°) a substance could be extracted with ether from the acidified solution which exhibited the ultraviolet absorption spectrum characteristic of 2-carboxypyrrole. Without preliminary alkali treatment no compound with any relevant absorption between 240 and 270 mμ. was obtained from the ether extract of the acidified solution (Fig. 3). On

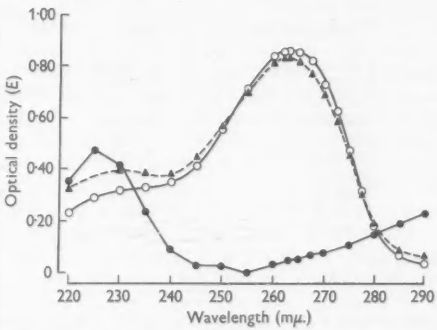



Fig. 3. Ultraviolet absorption spectra in ethanol of the ether-soluble material obtained from the alkali-treated product split off enzymically from bovine submaxillary-gland mucoprotein. ▲, 0·1 N-$Na_2CO_3$, 20 min., 100°; ○, 0·1 N-NaOH, 45 min., 100°; ●, no alkali treatment.

chromatographic analysis in the solvents described the compound obtained from the alkali-treated split product gave a single spot coinciding with synthetic 2-carboxypyrrole; the faster-running spot always present in the alkali hydrolysate of the complete mucoprotein was missing. No spot indicative of 2-carboxypyrrole was found in the untreated split product. The split product gave the direct Ehrlich reaction only on heating, whereas after alkali treatment the purple colour was formed instantly in the cold.

Analysis by chromatography and by light absorption in the ultraviolet of the material obtained by enzyme action on urine mucoprotein and subsequently treated with alkali (method 4) gave practically the same results as described in the preceding paragraph. The higher yield of 2-carboxypyrrole from enzyme action on urine mucoprotein (0·05 %) as compared with that from bovine submaxillary-gland mucoprotein (0·03 %) parallels the greater decrease in electrophoretic mobility with the former after RDE action (Curtain & Pye, 1955) under similar conditions.

*Possible precursors of 2-carboxypyrrole in biological material*

When the following substances were treated with 0·1 N-$Na_2CO_3$ for 20 min. at 100°, none produced material reacting with Ehrlich reagent: glutamic acid, glutamine, glutamine amide, glutaminyl-asparagine, 2-carboxy-5-pyrrolidone, 2-carboxypyrrolidine, 4-hydroxy-2-carboxypyrrolidine (hydroxyproline). *N*-Acetylglucosamine, after this treatment, is known to give a purple colour with Ehrlich reagent (Morgan & Elson, 1934); however, the ultraviolet absorption spectrum of the compound or compounds formed bore no similarity to that of the 2-carboxypyrrole control treated in the same manner (Fig. 4), confirming previous results

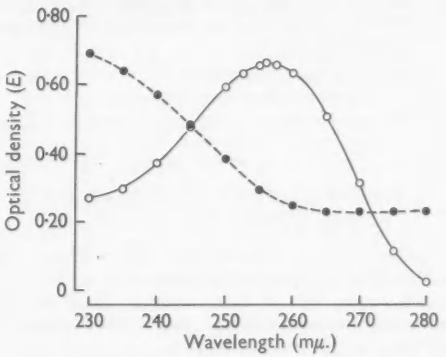



Fig. 4. Ultraviolet absorption spectra in 0·1 N-$Na_2CO_3$ of *N*-acetylglucosamine (●) and of 2-carboxypyrrole (○) treated with 0·1 N-$Na_2CO_3$ for 20 min. at 100°.

of Aminoff, Morgan & Watkins (1952). Chromatography, with butanol–pyridine–water as solvent, of the alkali-treated *N*-acetylglucosamine revealed a well-defined bluish-purple spot of $R_F$ 0·64, fading after a few hours; the 2-carboxypyrrole control gave a reddish-purple spot of $R_F$ 0·60, intensifying with time.

*Distribution of the directly Ehrlich-reacting substance(s) in mucoproteins, mucopolysaccharides and proteins*

Table 2 summarizes the results of testing 2% (w/v) solutions of various carbohydrate-containing and carbohydrate-free proteins and of an artificial mixture of galactose with a carbohydrate-free protein for the presence of a component or components reacting directly with Ehrlich reagent.

Table 2. *Distribution of the directly Ehrlich-reacting substance(s) in proteins, mucoproteins and mucopolysaccharides*

| Material | Molisch test | Direct Ehrlich reaction |
|---|---|---|
| Pepsin (twice cryst.) | Negative | Negative |
| Trypsin (twice cryst.) | Negative | Negative |
| Chymotrypsin (cryst.) | Negative | Negative |
| Insulin (cryst.) | Negative | Negative |
| Human globin (from haemoglobin) | Negative | Negative |
| β-Lactoglobulin (cryst.) | Faintly positive | Negative |
| Human-plasma albumin (five times cryst.), run 179, Dept. Phys. Chem. Harvard Medical School | Negative | Negative |
| Human-plasma albumin (five times cryst.) run 179, Dept. Phys. Chem. Harvard Medical School, plus galactose in a concentration of one-fifth of the protein present | Positive | Negative |
| Bovine plasma albumin (cryst.), Armour Laboratories, Chicago | Faintly positive | Faintly positive |
| Ovalbumin (cryst.) | Faintly positive | Negative |
| Human-urine mucoprotein (homogeneous) | Positive | Positive* |
| Human-sputum mucoprotein (homogeneous) | Positive | Positive* |
| Bovine submaxillary-gland mucoprotein (homogeneous) | Positive | Positive* |
| Sheep submaxillary-gland mucoprotein (homogeneous) | Positive | Positive* |
| Winzler's human-plasma mucoprotein (homogeneous) | Positive | Positive |
| Ovomucoid (purified) | Positive | Positive |
| Heparin (purified) | Positive | Negative |
| Hyaluronic acid (purified) | Positive | Negative |
| Chondroitin sulphuric acid (purified) | Positive | Negative |

* 2-Carboxypyrrole isolated.

*Interaction between 2-carboxypyrrole and reducing sugars in dilute mineral acid*

In Table 3 are recorded the colours formed by interaction of 2-carboxypyrrole and a reducing sugar under mild acid conditions. None of the reducing sugars produced any colour when 2-carboxypyrrole was omitted.

Table 3. *Colours resulting from heating an equimolar mixture of 2-carboxypyrrole (10 mg.) and a reducing sugar in 1·0 ml. 0·1 N-HCl for 30 min. at 100°*

| Sugar | Colour formed |
|---|---|
| — | Yellowish green |
| D-Glucose | Light brown |
| D-Galactose | Light brown |
| D-Fructose | Deep brown |
| Xylose | Deep brown |
| Ribose | Deep brown |

## DISCUSSION

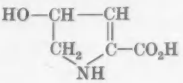
From the data presented it would appear that two preparations of 2-carboxypyrrole have been obtained from electrophoretically homogeneous mucoproteins (bovine submaxillary-gland and human-urine mucoprotein).

One preparation was obtained in a yield of about 0·05% of the mucoprotein after initial treatment of the protein with 0·64 N barium hydroxide for several hours at 100°; this material was contaminated with about 1% of another substance closely related to 2-carboxypyrrole (see below). Separation of 2-carboxypyrrole from the contaminant was achieved by eluting the former from a chromatogram of the material run in an appropriate solvent.

The other preparation was obtained from the low-molecular-weight compound released from the mucoproteins by the *Vibrio cholerae* enzyme RDE and heated subsequently with mild alkali. This preparation proved to be chromatographically homogeneous, uncontaminated by the second substance; the yield was 0·03–0·05% of the mucoprotein. Owing to the low yield there was not enough uncontaminated material available to crystallize 2-carboxypyrrole in order to get a melting point or to have an elementary analysis performed. However, it is thought that the evidence for the structure of this compound is strong

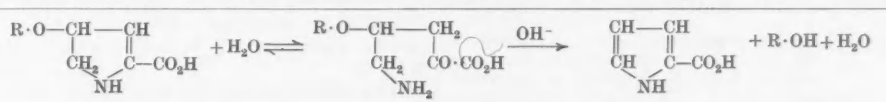
enough to permit its provisional identification as 2-carboxypyrrole. This evidence is as follows: chromatographic analysis in four different solvents of the pure material as obtained from mucoprotein and from the split product revealed coincidence in $R_F$ values and colour shade in the Ehrlich reaction with an authentic sample of 2-carboxypyrrole. The ultraviolet absorption spectra of pure material from both sources practically coincided between 220 and 290 m$\mu$. with that of authentic 2-carboxypyrrole. Catalytic reduction of the material from mucoprotein, which was contaminated with about 1% of another substance (see Results), caused the formation of a compound which on chromatographic analysis in four different solvents was found to be identical, in $R_F$ value and in the blue colour produced by isatin, with authentic 2-carboxypyrrolidine (proline). The contaminant was excluded as the source of proline.

(Gottschalk, 1954*b*) the position 4 was assigned to the hydroxyl group because of the biogenetic relationship to hydroxyproline. 2-Carboxy-4-hydroxypyrroline (I) is a labile structure. It could therefore be assumed that within the framework of the mucoprotein and in the split product stabilization is effected by engagement of the hydroxyl group of (I) in a glycosidic linkage with



(I)

an adjacent sugar residue, the presence of which in the split product was previously shown (Gottschalk, 1951). The resulting structure (II) in aqueous solution will undergo reversible ring-opening (Fig. 5); the open-chain product, as a $\beta$-alkoxy-



(II)

Fig. 5.

On the other hand, the ultraviolet absorption spectra of the untreated native mucoprotein and of the product split off from the mucoprotein by RDE action (pH 7·0, 37°) indicate that 2-carboxypyrrole is not present as such in the biological material. The finding that 2-carboxypyrrole, though not present in the enzymically released product, is readily formed from it on such mild alkali treatment as heating at 100° for 20 min. in 0·1 N sodium carbonate narrows considerably the range of substances which may be assumed as possible precursors. Compounds of the pyrrolidine class, e.g. proline and hydroxyproline, are stable to alkali treatment. Glutamine on very mild alkali treatment undergoes cyclization by forming an amide bond involving the α-amino group at the expense of the preformed one, resulting in the production of 2-carboxy-5-pyrrolidone and ammonia (Vickery, Pucher, Clark, Chibnall & Westall, 1935). Treatment of 2-carboxy-5-pyrrolidone with barium hydroxide at 100° causes hydrolytic cleavage of the ring, with the formation of glutamic acid (Menozzi & Appiani, 1894). When heated with 0·1 N sodium carbonate none of these compounds produced a substance reacting with Ehrlich reagent in the cold.

These negative results may suggest that the component of the mucoprotein and of the split product which on alkali treatment undergoes rearrangement to form 2-carboxypyrrole is a 2-carboxyhydroxypyrroline. In a preliminary note ketone, would be unstable to alkali (or acid), and reclosure of the ring concomitant with the expulsion of the sugar would yield 2-carboxypyrrole. The alkaline cleavage of glycosides of $\beta$-hydroxycarbonyl compounds is well known and the reaction mechanism is visualized as an elimination process rather than a hydrolysis (Ballou, 1954).

For the reason given an *O*-glycosidic linkage would appear to be more probable than an *N*-glycosidic linkage, as proposed earlier (Gottschalk, 1954*a*), when it was not known that 2-carboxypyrrole is not present as such in the mucoproteins.

When this paper was under review, a relevant note by Blix, Lindberg, Odin & Werner (1955) appeared. These authors give for sialic acid, a component of bovine submaxillary-gland mucoprotein (see Introduction), the provisional formula $C_{13}H_{21}O_{10}N$, $H_2O$ and find the acid to contain an *N*-acetyl group, an *O*-acetyl group, a primary alcohol group, an α-hydroxy group and a total of five hydroxyl groups. On treatment with concentrated sulphuric acid carbon monoxide is liberated and one group can be titrated with hypoiodite (Willstätter–Schudel); there is no indication of the presence of a glycosidic linkage. If, in Fig. 6, (III) (or its pyranose form $<2, 6>$) is envisaged as a possible structure of sialic acid, the relationship between this acid, the unstable 2-carboxy-4-hydroxypyrroline (open form) and 2-carboxypyrrole becomes evident. Indeed Fig. 6 would seem to account for all the observations made so

far at Blix's and at our laboratory. It is understood that it is only a scheme; thus the elimination of the tetrose may occur during or after the alkali-induced cyclization to the pyrrole.

The relevant facts about the point of attack of the *Vibrio cholerae* enzyme on suitable mucoproteins are, first, that it was shown for the urine mucoprotein (Perlmann, Tamm & Horsfall, 1952) and for the submaxillary-gland mucoprotein (Curtain & Pye, 1955) that their net negative charge decreased after treatment with influenza virus or with RDE and that amino groups of p$K$ value 10·8 to 11·2 were uncovered by RDE action on urine mucoprotein (Curtain, 1953). Secondly, it seems reasonable to assume that the rather strong basic group uncovered by RDE action and not counterbalanced by the loss in the split product of an equally strong basic group is masked in the native mucoprotein by an amide link with the carboxyl group of the precursor of 2-carboxypyrrole. However, more direct evidence is wanted to define RDE as an amidase.

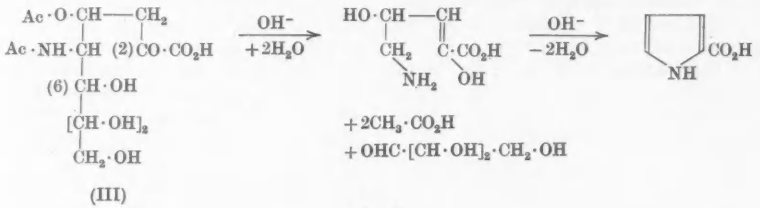



Fig. 6.

Concerning the fast-running compound producing with Ehrlich reagent a colour very similar to that given by 2-carboxypyrrole the following data are available: (*a*) the compound is formed only when the complete mucoprotein is submitted to treatment with barium hydroxide at 100°; it is not obtainable from the enzymically released product or from synthetic 2-carboxypyrrole on alkali treatment. (*b*) Treatment of the compound even with 25 % KOH for 20 hr. at 100° does not produce 2-carboxypyrrole. (*c*) It has an absorption maximum in the ultraviolet very close to, if not identical with, that of 2-carboxypyrrole. (*d*) It has in aromatic solvents a much higher $R_F$ value than that of 2-carboxypyrrole (Table 1). In butanol–pyridine–water the amide of 2-carboxypyrrole (kindly prepared by Dr J. W. Cornforth) has an $R_F$ value identical with that of the faster-moving compound; however, the colours formed with Ehrlich reagent and the absorption maxima in the ultraviolet differ significantly with the two compounds. (*e*) The compound does not react with ninhydrin and is somewhat volatile with water vapour. (*f*) In the total alkaline hydrolysate of mucoprotein there appears to be five times as much of this compound as of 2-carboxypyrrole (see Results).

From these data we have formed the working hypothesis that treatment of the mucoprotein with barium hydroxide or stronger alkali affects the segment of the mucoprotein comprising the precursor of 2-carboxypyrrole and the adjacent base (see above) in a twofold manner. First, about one in six amide bonds linking the carboxyl group of the precursor with the adjacent base will be cleaved, and the precursor, after rearrangement as in Fig. 6, will yield 2-carboxypyrrole. Secondly, cleavage of the remaining amide bonds will be forestalled by the alkali-induced rearrangement (by cyclization) of the base in such a manner as to form an alkali-resistant compound with 2-carboxypyrrole. Obviously, the enzymic cleavage of the amide bond, followed by alkali treatment of the split product, could not result in the formation of the faster-moving compound and was not found to do so.

According to this hypothesis the low yield of 2-carboxypyrrole after alkali treatment of the mucoprotein would result, in part at least, from the formation of a new compound involving more than 80 % of the 2-carboxypyrrole present, just as the low yield of 2-carboxypyrrole from the enzymically released product is partly due to the fact that only a fraction of the total amount of precursor present is split off. In addition, in both cases, the loss of 2-carboxypyrrole inherent in the method of purification will be a contributing factor.

It seems of interest that the precursor of 2-carboxypyrrole is present in all virus haemagglutinin-inhibitory mucoproteins prepared so far in a pure state and, if the direct Ehrlich reaction under the conditions described (Methods) is indicative of the presence of 2-carboxypyrrole, also in human plasma mucoprotein and ovomucoid (Table 2). No such compound was detectable in crystalline proteins free from carbohydrate or in mucopolysaccharides containing hexuronic acid, nor did an artificial mixture of carbohydrate-free protein and galactose give a positive direct Ehrlich reaction.

## SUMMARY

1. 2-Carboxypyrrole has been isolated from the alkali hydrolysate of homogeneous mucoproteins prepared from bovine submaxillary glands and from human urine. The imino acid, obtained in a yield of about 0·05%, was contaminated with approximately 1% of a substance of unknown structure. Separation of the two compounds was achieved by chromatography.

2. 2-Carboxypyrrole uncontaminated with the second compound, in a yield of about 0·03–0·05% of the mucoprotein, was obtained from the diffusible product released from the mucoprotein by the *Vibrio cholerae* enzyme (RDE) and treated subsequently with mild alkali.

3. Owing to its low yield the imino acid was not obtained crystalline. Its identification resides on the coincidence in chromatographic behaviour, in ultraviolet absorption spectrum and in proline production on catalytic reduction of authentic 2-carboxypyrrole and of the compound of biological origin.

4. The imino acid is not preformed in the biological material. Its precursor is, however, readily transformed into 2-carboxypyrrole when heated in 0·1N sodium carbonate solution for 20 min. at 100°.

5. Probable structures of the precursor are discussed.

6. The distribution of the precursor in proteins free of and associated with carbohydrate is described.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# *Clostridium oedematiens* η-Antigen, an Enzyme Decomposing Tropomyosin

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Oakley, Warrack & Clarke (1947) demonstrated the existence in culture filtrates of various strains of *Clostridium oedematiens* of six antigens, designated as α, β, γ, δ, ε and ζ. The α-antigen is produced by both type A and type B strains and is the main lethal agent in *Cl. oedematiens* toxic filtrates. Its biochemical action is unknown, but one of its physiological effects is that of a capillary poison (A. A. Miles & E. M. Miles, 1952; A. A. Miles & J. M. Elder, unpublished), which may well be due to an attack on some constituent of the capillary wall. Since the chemistry of the capillary wall is unknown, it was decided to find out if the crude toxin had any action on some known proteins, particularly those which, by association in lipoprotein structures, might themselves conceivably be concerned in some permeability phenomenon, in the hope of getting some clue to the nature of the toxic action.

It has been found that *Cl. oedematiens* type B (*gigas*) toxins contain an antigenic sulphydryl-activated enzyme decomposing tropomyosin. Serological tests show, however, that this enzyme is not identical with the lethal α-toxin, nor with any of the other previously described antigens. It has therefore been designated specifically as *Cl. oedematiens* η-antigen, and called a tropomyosinase. The characters of this enzyme are outlined below. Throughout this paper the word 'toxin' is used to denote the crude enzyme preparation, which was in fact toxic, and the specific antigens, distinguished in this toxin by serological tests, are denoted as α-antigen, β-antigen, etc.

## MATERIALS AND METHODS

*Toxins and antitoxins.* For examination of the biochemical properties of the enzyme, *Cl. oedematiens* toxin (type B, strain Albiston) batch Oe 8 was used. This was prepared in the Serum Department of this Institute by salting out from the culture filtrate with ammonium sulphate and drying *in vacuo*. A sample was dialysed at 2° in 7% solution in cellophan at pH 7·0 against 0·15 M-NaCl and the insoluble matter removed by centrifuging. The solution (20 ml.) was accurately dispensed in amounts of 0·02 and 1·0 ml. and freeze-dried, and the ampoules were filled with nitrogen and sealed. The analytical values per ml. of solution, tested after drying, were: 1·8 mg. total N; 1·4 mg. acid-soluble N; not more than 0·6 lecithinase units (Lewis & Macfarlane, 1953); no collagenase nor any haemolysin other than the lecithinase; and by a biological test approximately 50 $L_+$ units against the International Standard antitoxin. This preparation, Oe 8a, was arbitrarily designated as containing 150 units of tropomyosinase/ml.

For serological identification crude toxins of *Cl. oedematiens* and horse antisera prepared against them were obtained from several laboratories named below.

*Tropomyosin.* This was prepared from rabbit muscle substantially by the method of Bailey (1948, 1951). After the first fractionation with ammonium sulphate, the crude tropomyosin was dialysed against 0·1 M-KCl, precipitated with ethanol and dried, refractionated with ammonium sulphate and again precipitated with ethanol after dialysis. At this stage a 2% (w/v) solution in water was still faintly opalescent and 0·2 ml. gave a weakly positive test for tryptophan with Ehrlich's reagent. It was found convenient, owing to the time taken to dissolve dry tropomyosin, to make up at this stage a 2% solution in 0·1 M-KCl, dialyse overnight against the solvent, centrifuge clear and store the solution, now practically tryptophan-free, at −10°. The nitrogen content was determined on a sample; suitable quantities for experiment were withdrawn, diluted as desired and stored at 2° for up to a week without apparent change in viscosity.

*Myosin.* This was extracted from rabbit muscle according to Bailey (1942) and was used after one precipitation in 20 vol. water and resolution in 0·3 M-KCl at pH 7·0 and filtering through thick (Whatman no. 3) paper. This preparation, which probably contained some actin, is designated as (acto-)myosin as suggested by Bailey (1954).

*Buffers.* Stock solutions of phosphate buffer, $KH_2PO_4$–$Na_2HPO_4$, $I$ (ionic concentration) = 1·0 at pH 6·5, 7·0, 7·5 and 8·0, were diluted as required.

*Activators and inhibitors.* Solutions of cysteine hydrochloride and thiolacetic acid (both brought to pH 7·0 with NaOH), glutathione and sodium iodoacetate were made up just before use.

*Experimental mixtures.* In most of the experiments with tropomyosin mixtures were made of 1 vol. 1% protein in 0·1 M-KCl and 1·5 vol. of a mixture of toxin, cysteine and phosphate buffer in quantities adjusted to give a final concentration of 0·01 M cysteine and $I$ = 0·1; with (acto-)myosin $I$ was 0·3. The two components were placed 5 min. in a thermostat at 25° before mixing; the mixture was immediately transferred to a viscometer and readings were timed from the time of mixing.

*Viscosity measurements.* These were made in Ostwald viscometers, about 2·0 ml. in capacity with flow times 50–60 sec. for water at 25°, which were arranged in sets up to five with flow times within 5 sec. The relative viscosity, $\eta_{rel.}$, was taken as the ratio of the flow time of the sample to that of the solvent in the same viscometer. In kinetic

experiments the time of observation was recorded as that at the start of flow, i.e. no correction was made for the change in viscosity during the flow time. For the purposes of these experiments the results were not materially affected by the errors due to this change or by use of different viscometers.

*Nitrogen determinations.* These were made by micro-Kjeldahl technique. For determination of acid-soluble N, a measured volume of trichloroacetic acid was added to the sample to give a final concentration of 6·7% (w/v), and the mixture filtered after 10 min.

## EXPERIMENTAL

### *Action of* Clostridium oedematiens *type B toxin on tropomyosin*

Preliminary experiments showed that on incubating mixtures of tropomyosin and *Cl. oedematiens* toxin Oe 8a in phosphate–KCl solution in the presence of cysteine or thiolacetate the viscosity fell progressively to a limiting value, $\eta_{rel.}$ about 1·1. In the absence of toxin, the viscosity of the mixture decreased slowly but appreciably in the presence of thiolacetate, but was unaltered in the presence of cysteine; it was also unaltered in the presence of cysteine and boiled toxin, and in the presence of cysteine, toxin and homologous antitoxin. These facts indicated the presence of an antigenic SH-activated proteinase. In subsequent experiments cysteine was used as activator.

*Course of decomposition.* (1) *Rapid phase.* The toxin caused a rapid decrease of viscosity and then a slower formation of acid-soluble N from tropomyosin, illustrated in the following experiment:

Expt. 1. Toxin Oe 8a (125 units) in 12·5 ml. phosphate buffer (pH 7·0, $I = 0·1$) and 2·5 ml. 0·1 M cysteine was mixed at 25° with 10 ml. 1% tropomyosin in 0·1 M-KCl. A sample was placed in a viscometer and the remainder incubated in bulk at 25°, samples being removed at intervals for determination of the acid-soluble N. A control experiment with toxin previously heated at 100° for 5 min. showed no change. The results (Fig. 1) show that the relative viscosity had fallen nearly to the limiting value before there was an appreciable production of acid-soluble N. After 24 hr. at 25° only 50% of the tropomyosin N was acid-soluble; after dialysis in a cellophan bag at this stage for 72 hr. at 2° against 0·1 M-KCl, 70% of the tropomyosin N was still in the bag.

It seemed possible from this experiment that the decomposition was similar to that of myosin by trypsin, in which there is a rapid cleavage of the myosin molecule into two components only, H-meromyosin and L-meromyosin, of very different molecular weight, followed by a slow formation of acid-soluble N, which did not go to completion (Mihályi & Szent-Györgyi, 1953; Mihályi, 1953; Szent-Györgi, 1953). In two experiments similar to Expt. 1, the digested mixtures and the controls incubated without toxin were examined in the ultracentrifuge through the kindness of Dr R. A. Kekwick. There is some difficulty in stopping the action of this enzyme instantaneously. The bacterial proteinase is not inhibited by soya-bean trypsin inhibitor; iodo-acetate does not inhibit instantaneously; addition of excess of antitoxin may confuse the analysis; heating the tropomyosin at 100° in the experimental mixture without enzyme itself caused a small decrease in viscosity, e.g. from 2·5 to 2·3. The enzyme action was therefore stopped by cooling to 2° and dialysing, which removed the activator.

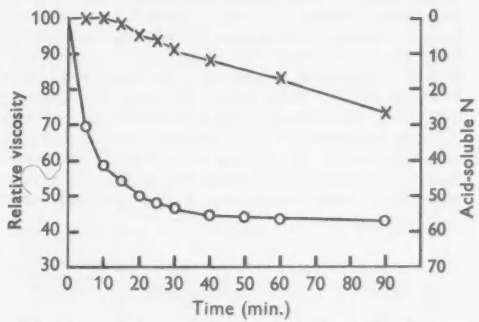


Fig. 1. Decomposition of tropomyosin by toxin Oe 8a: ○, relative viscosity as per cent of original value (2·54); ×, acid-soluble N formed as per cent of tropomyosin N.

The digestion was stopped in the first experiment (initial protein, 1%; $\eta_{rel.}$, 5·0) during the rapid phase ($\eta_{rel.}$, 1·8; acid-soluble N, < 10%) and in the second experiment (initial protein, 0·5%; $\eta_{rel.}$, 2·49) during the second phase ($\eta_{rel.}$, 1·09; acid-soluble N, 43%).

I am indebted to Dr Kekwick for his interpretation of these experiments. 'In the ultracentrifuge the tropomyosin showed characteristically a single sharp peak of the type generally displayed by highly asymmetrical molecules, with a sedimentation coefficient of $S_{20w} = 2·82$ at a concentration of 0·49 g./100 ml. ($I = 0·1$, pH 7·0). After treatment with the enzyme the sharp peak was replaced by a single rather broadly spreading peak having about the same mean sedimentation coefficient. This suggests a general breakdown of the tropomyosin molecule to a rather polydisperse product, which is in complete contrast to the behaviour of myosin towards trypsin (Mihályi & Szent-Györgyi, 1953). In this instance the breakdown of myosin led to the formation of two well-defined components quite different in the ultracentrifuge from myosin itself.'

(2) *Slow phase.* Mihályi (1953) found that the further decomposition of meromyosin by trypsin

was inhibited by the products of the reaction, and that the rate was proportional to the square root of the enzyme concentration. The slow phase of decomposition of degraded tropomyosin by the *Cl. oedematiens* enzyme was examined as follows:

Expt. 2. Toxin Oe 8a (150 units, 1·8 mg. N) dissolved in 14 ml. phosphate buffer (pH 7·0, $I = 0{\cdot}1$) and 6 ml. 0·03 M cysteine was incubated at 25° with 40 ml. 0·9% tropomyosin (59 mg. N) in 0·1 M-KCl. When the relative viscosity had fallen from 3·5 to 1·4, the mixture was rapidly heated in a boiling-water bath and kept there for 6 min. After cooling, the relative viscosity had fallen to 1·22, presumably partly because the enzyme was not inactivated instantaneously. (It was ascertained in a separate experiment that the rate of formation of acid-soluble N was the same in heated and unheated solutions.) The mixture (56 ml.) was dialysed for 20 hr. at 2° against 0·1 M-KCl; the recovered solution contained all the original tropomyosin N, of which 10·3% was now acid-soluble. Three flasks *A*, *B* and *C* containing 15·0 ml. of this substrate and respectively 75, 37·5 and 15 units enzyme in 5·0 ml. phosphate–cysteine (pH 7·0) were incubated at 38° and the acid-soluble N was determined at intervals. Fig. 2 shows that at the highest concentration of enzyme more than 90% of the total N had

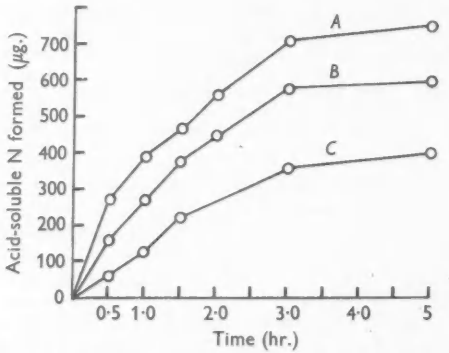


Fig. 2. Production of acid-soluble N from degraded tropomyosin. Enzyme concn. (units/ml.): *A*, 3·75; *B*, 1·87; *C*, 0·75. Total N 770 μg./ml.

become acid-soluble in 5 hr.; the curves with lower concentrations of enzyme indicate that in time the enzyme becomes inactivated in these conditions. The rates for the first 30 min. were in the ratio 4·2:2·4:1 for enzyme concentrations in the ratio 5:2·5:1; this indicates that the rate is proportional to the enzyme concentration directly, rather than its square root, as the two higher rates were probably not linear for the full 30 min. period. After 22 hr. at 38°, 10 ml. mixture *A* was dialysed in a cellophan bag at 2° against changes of 0·1 M-KCl for 24 hr., at which point 63% of the original tropomyosin N was still in the bag.

In the next experiment repeated additions of toxin were made to find out if the protein could be completely converted into diffusible products:

Expt. 3. Tropomyosin (10·0 ml., 15·2 mg. N) was incubated at 25° with 75 units enzyme in 5·0 ml. SH-buffer (pH 7·0); after 5 hr. another 5·0 ml. of enzyme was added. After 20 hr. the mixture was dialysed for 24 hr. and analysed; it contained 78% of the original protein N. A sample of this solution (7·5 mg. N) was re-incubated at 25° with another 150 units enzyme in SH-buffer for 21 hr., then dialysed and analysed. At least 40% and not more than 55% of the original tropomyosin N was still non-diffusible. Owing to the slow rate and the large correction for the toxin N added a further attempt to determine the end point with crude enzyme seemed unprofitable.

It appears that the decomposition of tropomyosin by *Cl. oedematiens* type B toxin takes place in three stages: (1) a rapid phase, with decrease of viscosity, probably due to a simultaneous attack at several points of the molecule with formation of particles still large enough to be insoluble in trichloroacetic acid; (2) a second phase in which these particles are broken down to particles soluble in trichloroacetic acid with some products diffusible through cellophan; (3) a very slow production of diffusible N from the remainder. The first stage of viscosity reduction is practically completed before the second phase begins; the overlapping in the second and third stages has not been accurately determined. It is not certain that only one enzyme is concerned.

### *Action of the toxin on (acto-)myosin*

The action of the toxin on (acto-)myosin is similar to that on tropomyosin, with a decrease of viscosity in the first phase and then a slow pro-

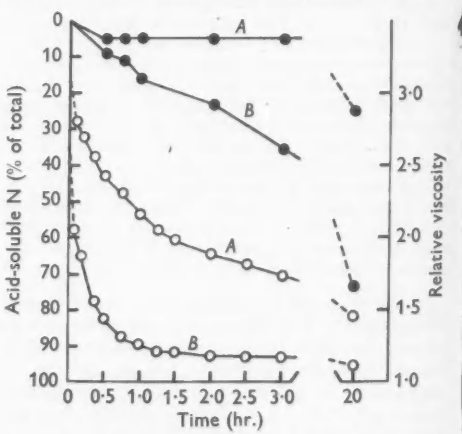


Fig. 3. Decomposition of myosin and tropomyosin. Toxin Oe 8a, 3·45 units/ml., pH 7·0; *A*, myosin (0·86 mg. N/ml., $I = 0{\cdot}3$); *B*, tropomyosin (0·91 mg. N/ml., $I = 0{\cdot}1$). Time scale interrupted at 3 hr. ○, viscosity relative to solvent; ●, acid-soluble N as per cent of total.

duction of acid-soluble N. The early phase is shown in Fig. 3. After 20 hr. incubation at 25° the relative viscosity of the (acto-)myosin had fallen to 1·46, compared with 1·1 for tropomyosin, and the acid-soluble N was 25% with (acto-)myosin, against 74% with tropomyosin. On re-incubating these mixtures with additional enzyme (final concentration, 1·5 units/ml.) the viscosity of the (acto-)myosin was not further decreased, but the acid-soluble N rose slowly in 22 hr. to about 50%, compared with over 90% for tropomyosin. At this point about 60% of the (acto-)myosin N was still non-diffusible, of which only about 10% was acid-soluble, while about 40% of the tropomyosin N was non-diffusible but mainly acid-soluble. This experiment compares the extent of the digestion of the two proteins by the enzyme; it is not intended as a comparison of the rate of attack, as at $I = 0{\cdot}1$ tropomyosin still shows some degree of linear aggregation (Tsao, Bailey & Adair, 1951) and the decrease in viscosity may be due partly to some disaggregation consequent on the splitting of peptide bonds.

*Properties of the tropomyosinase*

The general properties were examined by measurement of the viscosity-reducing action on tropomyosin.

*Unit of enzyme activity.* Since the purity of the substrate cannot readily be assessed and its viscosity may vary in different batches no attempt was made to define a unit of enzymic activity in terms of the absolute amount of substrate decomposed. For the present experiments it was convenient to designate the stable preparation of 0·02 ml. freeze-dried Oe 8a as a standard containing 3 units and compare other activities with this, using the same preparation of substrate in similar conditions, which varied slightly with the purpose of the experiment. The activity of 1 unit in terms of substrate decomposed may be gauged from the following: 1 unit of Oe 8a in a total vol. 2·5 ml. with 0·01 M cysteine at pH 7·0 and 25° and 0·4% tropomyosin decreased the relative viscosity to about 82% of its initial value in 30 min., i.e. approximately 1 unit degraded 2 mg. tropomyosin in these conditions.

*Optimum* pH. As the viscosity of the tropomyosin mixtures increased slightly with increase in pH, the optimum pH was determined approximately for the crude enzyme by comparison of the time taken by 1 unit of enzyme at a given pH to decompose 25% of the substrate at that pH. Table 1 shows that the optimum was about 7·0 and subsequent experiments were made at this pH.

*Effect of enzyme concentration on rate of action.* Mixtures were made containing 0·2, 0·4 and 0·8 ml. toxin (6·7 units/ml.) in 0·1 M-KCl, 0·25 ml. 0·1 M cysteine, 0·45 ml. phosphate buffer (pH 7·0), 0·1 M-KCl to total volume 1·5 ml.; and then 1·0 ml. of 1% tropomyosin at 25°. The subsequent decrease in viscosity at 25° is shown in Fig. 4. From these curves Fig. 5 was constructed, showing that

Table 1. *Optimum* pH *of tropomyosinase*

The mixtures contained 1·0 ml. tropomyosin (1% or 0·75%). 1·0 ml. phosphate buffer ($I = 0{\cdot}1$), 0·25 ml. 0·1 M cysteine and 0·25 ml. toxin (1 unit) or 0·1 M-KCl.

| pH | $\eta_{rel.}$ without toxin at substrate concn. 0·4% (*a*) | 0·3% (*b*) | Time taken with toxin to reduce $\eta_{rel.}$ from (*a*) to (*b*) 0·3% concn. (min.) |
|---|---|---|---|
| 6·5 | 2·36 | 1·89 | 60 |
| 7·0 | 2·44 | 1·90 | 47 |
| 7·5 | 2·54 | 1·95 | 55 |
| 8·0 | 2·58 | 2·03 | 60 |

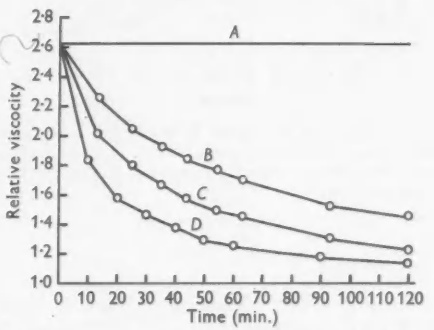


Fig. 4. Time curve of viscosity reduction with different enzyme concentrations. Total vol., 2·5 ml.; tropomyosin, 0·4%; vol. of toxin (6·7 unit/ml.) added: *A*, nil; *B*, 0·2 ml.; *C*, 0·4 ml.; *D*, 0·8 ml.

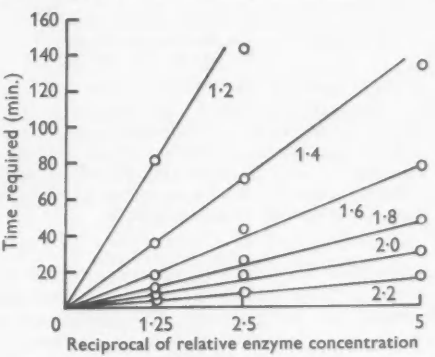


Fig. 5. Inverse relation between time required to reach a given viscosity and the enzyme concentration. Data from expt. in Fig. 4. The relative viscosity is marked on each curve.

the time taken to reach a given value of $\eta_{rel.}$ is proportional to the reciprocal of the concentration of the enzyme. With the higher concentrations of enzyme the early readings are more subject to error owing to the rapidity of the change, and with low concentrations the times for the lower $\eta_{rel.}$ values are more subject to error because of the slowness of the rate.

*Activation and inhibition.* The enzyme was activated by cysteine, glutathione (Fig. 6) and

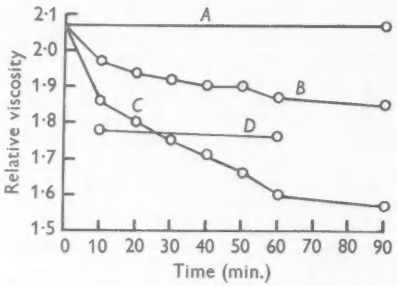


Fig. 6. Activation of enzyme by sulphydryl compounds. Enzyme, 1 unit; tropomyosin, 0·37%. *A*, no activation; *B*, glutathione 0·004 M; *C*, cysteine 0·01 M; *D*, thiosulphate, 0·01 M.

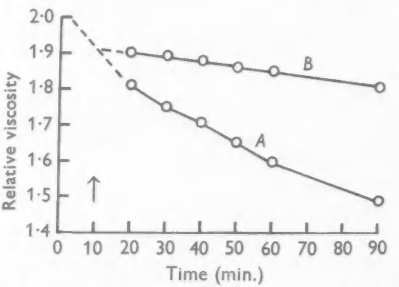


Fig. 7. Reversal of activation with cysteine by iodoacetate. Toxin, 1·0 unit; 0·25 ml. 0·1 M cysteine; 1·0 ml. tropomyosin; phosphate buffer to 2·0 ml. initial vol. Incubated at 25°. At arrow (10 min.) 0·5 ml. buffer added to *A*, and 0·5 ml. 0·1 M iodoacetate to *B*, and the mixtures transferred to viscometer at 25°.

thiolacetate. Thiosulphate did not activate the enzyme but the viscosity of the tropomyosin was decreased in its presence. The activation by cysteine was diminished by iodoacetate, but with a calculated excess the inhibition was not complete (Fig. 7).

Crystalline soya-bean trypsin inhibitor (Worthington Biochemical Sales Co., New Jersey) did not inhibit the tropomyosinase in a ratio calculated to be at least 6:1 on the polypeptide content (N × 6) of the crude toxin, and presumably much greater on the actual enzyme. This sample of inhibitor had been found to inhibit four times its weight of crystalline trypsin (personal communication from Mrs Sneath).

*Action on collagen and red blood cells.* Using 'azocoll' as substrate, with a technique similar to that of Bidwell & van Heyningen (1948), no collagenase was detected in the preparations Oe 4 and Oe 8a; positive results were obtained with smaller amounts of comparable crude *Cl. welchii* toxin. The preparations Oe 4 and Oe 8a had no haemolytic activity towards horse or sheep cells in phosphate buffer in the presence of cysteine (i.e. in conditions excluding the action of the $Ca^{2+}$-activated lecithinase); this indicated the absence from these preparations of any SH-activated haemolysin similar to *Clostridium welchii* $\theta$-toxin and of the *Cl. oedematiens* $\zeta$-haemolysin described by Oakley *et al.* (1947).

### *Antigenic nature of* Clostridium oedematiens *tropomyosinase*

*Inhibition by antisera.* The tropomyosinase activity was inhibited after allowing the enzyme to react with certain horse antisera to *Cl. oedematiens* type B toxins. An antitoxic serum (no. 1141 from the Wellcome Research Laboratories) was designated as a standard serum containing 6000 units of antitropomyosinase/ml. and the relative titres of other sera were determined in the following way.

Toxin and antisera were dissolved or diluted in phosphate buffer (pH 7·0, $I = 0·1$). In a series of tubes the test dose of toxin Oe 8a (1 unit tropomyosinase in 0·5 ml.) + 0·25 ml. 0·1 M cysteine was mixed with antitoxin and the volume made up to 1·5 ml. with buffer. After 25 min. at room temperature the tubes were placed at 25° for 5 min.; 1·0 ml. 1% tropomyosin in 0·1 M-KCl was added, and the mean residual enzyme activity determined, in relation to that of the test dose without any antitoxin, by the times required to decrease the viscosity to three or four successive levels, e.g. from initial $\eta_{rel.}$ 2·4 to 2·2, 2·1, 2·0 and 1·9. The curve relating residual activity to antitoxin units was approximately linear and reasonably reproducible between 0·7 and 1·4 units antitoxin. In seven separate tests, the residual activity of 1 unit of enzyme (Oe 8a) after combination with 1 unit antitropomyosinase (Serum 1141) was 0·54, 0·58, 0·58, 0·34, 0·58, 0·58 and 0·62 unit. With lower or higher amounts of antitoxin (less than 0·6 or more than 1·4 units) the results were less reproducible, presumably owing to the errors in the steep or flat part of the activity curve respectively. In practice the titre of an unknown serum was first determined approximately and redetermined within the range 0·7–1·4 units by reference to the standard antitoxin curve.

Inspection of the titres of various sera (Table 2) shows first that there is no correlation between the

antitropomyosinase and the anti-α-toxin titres; and secondly that there is no correlation between the antitropomyosinase and the anti-ζ-toxin titres. All the antisera to *Cl. oedematiens* type B toxins contained some antitropomyosinase, although the titres of the two sera produced against the type B Todd strain were relatively low; a sample of *Clostridium haemolyticum* antitoxin was also inhibitory. The *Cl. oedematiens* International Standard antitoxin and the type A antitoxin 644 and *Cl. welchii* antitoxin had no demonstrable antitropomyosinase; because of the increase in initial viscosity of the test mixtures by the addition of glycerol (International Standard) or serum protein the limiting values of low-titre sera could not be determined accurately.

*Tropomyosinase content of various crude toxins*

The tropomyosinase content of different preparations was estimated by comparing the activity of a sample with that of 1 unit of the standard Oe 8a in conditions similar to those for the antitoxin titration, but omitting the 25 min. period for combination. The results in Table 3 show that the

Table 2. *Titres of specific antitoxins in antitoxic horse sera*

The antitropomyosinase (anti-η) titres were determined against a test dose of *Cl. oedematiens*, type B toxin Oe 8a.

| | Sera | | Titres of specific antitoxin (units/ml.) | | |
|---|---|---|---|---|---|
| Immunizing toxin *Cl. oedematiens* | Source | No. | Anti-η* | Anti-α† | Anti-ζ‡ |
| Type B, Albiston | W.R.L. | 1141 | 6000 | 1000 | 5000 |
| | W.R.L. | 2209 | 280 | 1200 | 2500 |
| | W.R.L. | 1152 | 4800 | 1000 | 3000 |
| | L.I.P.M. | 77 | 8000 | 3000 | — |
| | L.I.P.M. | Monospecific§ | 540 | 1000 | — |
| Type A + B | W.R.L. | 850 | 1650 | 1750 | 5000 |
| Type B, Todd | W.R.L. | T3 | 1600 | 6250 | 110 |
| | W.R.L. | T61 | *ca.* 80 | 1650 | 150 |
| Type A | R.W.L. | 644 | (Nil) | 800 | 140 |
| | M.R.C. | Standard | (Nil) | 20 | — |
| *Cl. haemolyticum* | W.R.L. | EX 1250 | 140 | — | — |
| *Cl. welchii* | L.I.P.M. | RP586§ | (Nil) | 2500‖ | — |
| | L.I.P.M. | Monospecific§ | (Nil) | 1000‖ | — |

W.R.L. = Wellcome Research Laboratory. L.I.P.M. = Lister Institute of Preventive Medicine.
M.R.C. = National Institute for Medical Research, Mill Hill.

* Serum no. 1141 as standard. † Anti-lethal *Cl. oedematiens* i.u. ‡ Wellcome units.
§ Pepsin-refined antitoxin. ‖ *Cl. welchii* antitoxin i.u.

Table 3. *Tropomyosinase content of various crude toxins*

| Kind of toxin: Organism | Type | Strain | No. | Units tropomyosinase | Source of toxin |
|---|---|---|---|---|---|
| *Cl. oedematiens* | B | Albiston | Oe 7 | 0·92/mg. | L.I.P.M. |
| | | | Oe 8 | 1·20/mg. | |
| | | | OC 12345 | 0·88/mg. | W.R.L. |
| | | | OK 15145 | 0·42/mg. | |
| | B | *gigas* | XE 1074 | 0·33/mg. | |
| | B | Todd | 12XB | Nil in 7·0 mg. | |
| | B | — | AE 1258* | Nil in 1·0 ml. | |
| | | | | Nil in 10 ml. after concentration | |
| | A | — | OC 19345 | 0·02/mg. | |
| | A | — | NX 510 | Nil in 20 mg. | |
| | A | — | Test toxin | Nil in 10 mg. | M.R.C. |
| | A | — | Evans | Nil in 10 mg. | D. G. Evans |
| *Cl. haemolyticum* | — | — | NX 605 | 0·05/mg. | W.R.L. |
| | | | HC 231048 | 0·02/mg. | |

* Liquid culture filtrate.

crude toxins of *Cl. oedematiens* type B, strains Albiston or *gigas*, are the highest in activity. No activity was found in the type A toxin, NX 510, nor in two other *Cl. oedematiens* toxins, the M.R.C. test toxin and one given by Dr D. G. Evans, which are probably also type A, nor was any activity found in various samples of *Cl. welchii*, *Clostridium septicum* and *Corynebacterium diphtheriae* toxins.

The *Cl. haemolyticum* toxin tested had some activity. The relative inhibitory activity of the *Cl. oedematiens* antiserum no. 2209 and the *Cl. haemolyticum* antiserum EX 1250 against a test dose of the standard toxin Oe 8a and a test dose of the *Cl. haemolyticum* toxin, each containing 0·5 unit enzyme, were about equal. It seems probable by the equality of the cross-neutralization that the antigens are identical. This is of some taxonomic interest, as *Cl. oedematiens* type B and *Cl. haemolyticum* toxins also possess the $\beta$-antigen (lecithinase) in common (Oakley *et al.* 1947; Macfarlane, 1950).

*Designation of the tropomyosinase*

It is concluded that the tropomyosinase in the *Cl. oedematiens* type B filtrates is a specific antigen distinct from any of those designated by Oakley *et al.* (1947), on the following grounds: (1) By the lack of correlation in antitoxin titres of different antisera, it is distinct from the $\alpha$- and $\zeta$-toxins. (2) It is also distinct from the $\alpha$-toxin, because it was not detected in the M.R.C. toxin used for titration of the International Standard antitoxin, nor in the lethal type A toxin NX 510. (3) It is distinct in biochemical activity from the $\beta$- and $\gamma$-antigens, which are lecithinases, and from the $\alpha$- and $\zeta$-antigens, which are haemolytic. (4) It is distinct by distribution and activity from the $\epsilon$-antigen, which, possibly as a lipase, gives rise to a pearly layer in egg yolk and which is found in type A but not in type B culture filtrates.

The antigen has therefore been designated as *Cl. oedematiens* $\eta$-antigen. From the biochemical point of view, it seems convenient to name the enzyme in the meantime as a tropomyosinase until its substrate specificity is clearly defined.

## DISCUSSION

The formation *in vivo* of an enzyme which decomposes (acto-)myosin and tropomyosin might be of some significance in disease caused by *Cl. oedematiens*. Curiously enough, tropomyosinase does not appear to be produced by type A strains, which in man cause gas gangrene due to infection of muscle, but it is produced by type B strains which cause infectious necrotic hepatitis (black disease, German braxy) in sheep; in this disease the organisms multiply in the liver, in necrotic areas produced by invasion with liver flukes (Turner, 1930). There is little doubt that the main lethal component in type A and type B toxins produced *in vitro* is the classical $\alpha$-toxin, but the significance in infections of the other antigens, including the tropomyosinase, has not been assessed.

It does not appear that the presence of any proteinase in *Cl. oedematiens* filtrates has previously been established. Evans (1947) examined filtrates from a number of strains for collagenase and gelatinase but, as in the present work, with negative results. Taxonomically it is of interest that the *Cl. haemolyticum* toxin and antitoxin examined contained tropomyosinase and the anti-enzyme respectively, and that cross-neutralization tests indicate that the *Cl. haemolyticum* and *Cl. oedematiens* antigen are identical. Oakley *et al.* (1947) pointed out that the possession in common of the $\beta$-antigen supported the view that *Cl. haemolyticum* is a member of the *oedematiens* group, and this view is again strengthened by the common possession of the $\eta$-antigen.

The crude tropomyosinase preparations decompose tropomyosin in at least two distinct stages, characterized by a rapid decrease in viscosity and a slower but complete decomposition to particles soluble in trichloroacetic acid. It also decomposes (acto-)myosin with production of at least 50% of acid-soluble nitrogen. Bailey (1948) suggested that tropomyosin may be one of the ultimate units of which myosin is composed, though it is certain that myosin consists of something more than tropomyosin; for one thing tryptophan is present in myosin but not in tropomyosin. The present findings that (acto-)myosin and tropomyosin are both decomposed by the bacterial preparation is at least not inconsistent with Bailey's suggestion, and the experiments indicated the possibility of a completely or relatively resistant 'core' in (acto-)myosin. The end point of this action was not established, however, nor has it yet been shown that the decomposition of tropomyosin and (acto-)myosin is due to the same or to a single enzyme. The tropomyosinase may be a useful tool in the elucidation of the structure of these proteins, but considerable purification of the enzyme would first be necessary.

## SUMMARY

1. Certain crude toxins of *Clostridium oedematiens* decompose tropomyosin and myosin.

2. The decomposition of tropomyosin takes place in three stages characterized by: (1) rapid decrease in viscosity; (2) production of particles soluble in trichloroacetic acid but not diffusible through cellophan; (3).slow production of diffusible particles.

3. The viscosity-reducing action is due to a sulphydryl-activated enzyme, which has been named a tropomyosinase, whose action is inhibited by homologous antisera.

4. The enzyme does not attack collagen, nor haemolyse the red blood cells of sheep and horses. It is activated by cysteine, glutathione and thiolacetate, but not by thiosulphate. It is not inhibited by soya-bean trypsin inhibitor.

5. By neutralization tests with selective antisera, this tropomyosinase was identified as a specific antigen which has been designated, in accordance with the nomenclature of Oakley *et al.* (1947), as *Cl. oedematiens* $\eta$-antigen. It has been found in crude toxins of *Cl. oedematiens* type B and *Clostridium haemolyticum*, but not in *Cl. oedematiens* type A nor in *Clostridium welchii*, *Clostridium septicum* or *Corynebacterium diphtheriae* toxins.

I wish to express my thanks to my colleagues Mr Weitz and Dr Dolby, to Miss M. Barr, Dr D. A. Long, Dr D. G. Evans and especially to Dr G. H. Warrack of the Wellcome Research Laboratories for gifts of toxins and antitoxins, to Miss S. Jolley for her assistance, and to Mr H. Murray for the ultraeentrifugal examinations.

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# The Interconversion of Serine and Glycine: Participation of Pyridoxal Phosphate

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The enzyme system of avian and mammalian liver which catalyses the reversible synthesis of serine from glycine and formaldehyde has been shown to require tetrahydropteroylglutamic acid (tetrahydroPGA) as a coenzyme (Blakley, 1954*b*, *d*; Kisliuk & Sakami, 1954). In well dialysed, unfractionated liver extracts pteroylglutamic acid (PGA) and some other derivatives of it besides tetrahydroPGA also stimulate serine synthesis (Blakley, 1954*a*, *d*), but the evidence indicates that in such crude enzyme preparations PGA and derivatives of it other than tetrahydroPGA are first converted enzymically into the latter compound before participating in serine biosynthesis (Blakley, 1954*d*). When crude enzyme preparations are used diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP) in conjunction with PGA also stimulate serine–glycine interconversion, but the effect of ATP and DPN appears to be concerned with the transformation of PGA into tetrahydroPGA (Blakley, 1954*d*). Thus the only coenzyme of serine biosynthesis, for which *in vitro* studies by the isotope method have provided conclusive evidence, is tetrahydroPGA. *In vivo* experiments have suggested, however, that pyridoxal phosphate also participates in serine synthesis (Deodhar & Sakami, 1953).

Serine biosynthesis in the presence of a partially purified enzyme system has now been studied by a manometric method, and under appropriate conditions a marked activation by pyridoxal phosphate has been observed (Blakley, 1954*c*). In the present communication the evidence for the activation of enzymic serine synthesis by pyridoxal phosphate is presented in detail, together with data concerning the effect on the reaction of other pyridoxine derivatives.

## EXPERIMENTAL

*Reagents.* TetrahydroPGA was prepared by catalytic hydrogenation of neutral aqueous solutions of commercial preparations of PGA (O'Dell, Vandenbelt, Bloom & Pfiffner, 1947). Pyridoxine derivatives were gifts: crystalline pyridoxal phosphate (4-formyl-3-hydroxy-2-methylpyrid-5-ylmethyl dihydrogen phosphate) was received from Dr A. L. Morrison of Roche Products and crystalline pyridoxamine 5-phosphate (4-aminomethyl-3-hydroxy-2-methylpyrid-5-ylmethyl dihydrogen phosphate), pyridoxine 5-phosphate (3-hydroxy-4-hydroxymethyl-2-methylpyrid-5-ylmethyl dihydrogen phosphate) and deoxypyridoxine 5-phosphate (3-hydroxy-2:4-dimethylpyrid-5-ylmethyl dihydrogen phosphate) from Dr H. A. Sober.

*Animals.* Various strains of rabbit were used, though the majority were the wild type. Some had been infected with myxomatosis and had recovered. The enzyme content of the liver varied considerably and appeared to be dependent on the general condition of the animal, but not on the particular strain of animal used. Animals were kept on a stock diet before use, the period on the diet ranging from 24 hr. to many months.

## METHODS

*Determination of enzyme activity.* Under the experimental conditions employed, serine synthesis proceeded at a constant rate, proportional to the amount of enzyme added, until the formaldehyde (added in smaller amount than glycine) was almost exhausted (unpublished observations). Enzymic activity was therefore assayed by estimating the serine formed in 1 hr. under standard conditions. The enzyme preparation in the presence of tetrahydroPGA (0·0013 M) and bicarbonate (0·01 M) was equilibrated with $N_2+CO_2$ (95:5) (v/v) at 37° in Warburg vessels. When equilibration was complete glycine and formaldehyde were tipped in from the side arm to give a final concentration of 0·1 and 0·01 M respectively. After 1 hr. the manometers were removed from the bath, the water was blotted from around the necks of the flasks and the side-arm stoppers, the flasks were removed from the manometers and a glass sphere was placed in the neck of each flask. The flasks were immediately placed in a boiling-water bath and kept there for 2 min. to inactivate the enzyme. When the flasks had cooled, 0·3 ml. of 0·5 M sodium periodate (adjusted to pH 6·0) was placed in the side arm of each. The flasks were then replaced on their manometers, flushed with pure $CO_2$ and equilibrated at 37°. When the periodate was tipped into the main compartment, serine was oxidized with the evolution of 1 mole $CO_2$/mole of serine (Fig. 1). The reaction was completed in 20 min. Only small amounts of $CO_2$ (about 3 $\mu$moles) were evolved by reactions other than the oxidation of serine by periodate, and an estimate of this 'blank' $CO_2$ production was obtained by carrying out the determination with the omission of tetrahydroPGA. When crude enzyme preparations were used some serine was produced even in the absence of added tetrahydroPGA. In such cases both formaldehyde and tetrahydroPGA were omitted from the control flask, but even under these conditions the 'blank' was often too high, owing to some serine synthesis proceeding even in the absence of tetrahydroPGA and formaldehyde. Consequently, values for the activity of crude enzyme preparations were frequently low.

From 1 to 30 $\mu$moles of serine added to the enzyme-assay system could be recovered with an accuracy of $\pm 5\%$ (Fig. 1). Specific activity of the enzyme is expressed as $\mu$moles serine formed/hr./mg. protein under standard conditions. A unit of enzyme is taken as that amount forming 1 $\mu$mole serine/hr. under standard conditions.

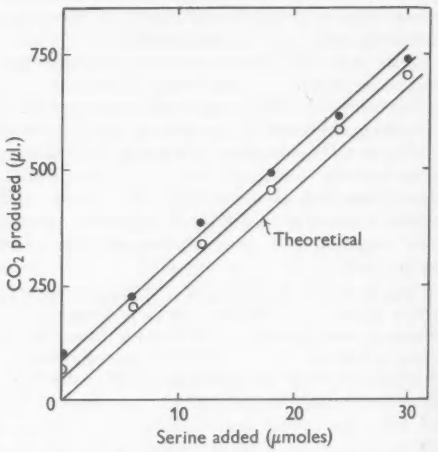


Fig. 1. Production of $CO_2$ under standard assay conditions by periodate oxidation of pure serine added to complete enzyme system, ●; and of serine added to the same system with enzyme omitted, ○.

*Preparation of the enzyme.* The rabbits were killed by a blow on the head and the livers quickly removed and dispersed in acetone as previously described (Blakley, 1954*d*). After drying at room temperature and about $3 \times 10^{-2}$ mm. Hg for 2 hr. over $P_2O_5$ the powder was extracted with 15 times its weight of cold 0·01 M potassium phosphate buffer (pH 7·5) for 1 min. in a Waring Blendor. The extract was allowed to stand for 30 min. at 5° before centrifuging (1 hr. at 4300 *g*) at 2°. The supernatant was brought to 53° within 5 min., held at this temperature for a further 10 min. and then rapidly cooled in ice water. Centrifuging at 4300 *g* for 30 min. produced a clear supernatant, which was fractionated with ammonium sulphate (adjusted to pH 7·5). The fraction precipitating between 1·47 and 2·06 M ammonium sulphate was collected separately, dissolved in a small volume of 0·01 M phosphate (pH 7·5) and dialysed for 3 hr. in a rocking dialyser in the cold (5°) against 100 vol. 0·02 M potassium phosphate buffer (pH 7·5) and then for a further 3 hr. against 0·001 M phosphate (pH 6·3). Attempts at further purification of the enzyme by the usual methods were attended by considerable losses, and difficulty was experienced in reproducing the fractionation from one batch of enzyme to another. The best results from ethanol fractionation were obtained if a trial fractionation of a small portion of the enzyme was carried out first. A small portion of the dialysed solution was adjusted to pH 6·2 with 1 M acetate (pH 5·0), diluted to give a protein concentration of about 12 mg./ml. and fractionated with ethanol, the temperature being lowered

Table 1. *Summary of yields and specific activities of fractions obtained during the purification of serine-synthesizing enzyme from rabbit-liver–acetone powder*

The details given are for a preparation from 53·5 g. acetone powder. Units of activity are expressed as μmoles serine/hr. Specific activity is expressed as μmoles serine/hr./mg. protein.

| Fraction | Volume (ml.) | Total protein (mg.) | Total units | Specific activity |
|---|---|---|---|---|
| Phosphate-buffer extract | 644 | 18 900 | 54 000* | 1·9* |
| Supernatant after heating at 53° | 504 | 10 900 | 47 500* | 2·8* |
| Ammonium sulphate precipitate | 63 | 3 270 | 78 200 | 16·5 |
| Ethanol-precipitated fraction | 57 | 608 | 27 400 | 27·4 |

* Estimation of activity in these fractions gave low values, probably because some synthesis occurred in the absence of added tetrahydroPGA and formaldehyde, and hence was included in the blank.

from 0 to −3° during addition of ethanol. Five fractions were collected over the range of ethanol concentration 0–25% (v/v), and each fraction was tested for enzymic activity. The remainder of the enzyme was then fractionated with ethanol in the same way and the most active fraction, as previously determined in the trial, was dissolved in cold 0·01 M phosphate buffer (pH 7·5). To preserve activity it was found necessary to precipitate the enzyme immediately with 2·21 M ammonium sulphate (adjusted to pH 7·5), centrifuge after standing 10 min. at 0°, and redissolve the precipitate in dilute phosphate buffer (pH 7·5). With some preparations the enzymic material could be further purified by ammonium sulphate fractionation at pH 7·5, the material precipitating at ammonium sulphate concentration 1·54–2·02 M being collected separately and dissolved in a small volume of cold 0·01 M phosphate (pH 7·5). At any stage in the preparation the enzyme solution could be kept frozen without great loss in activity, but considerable and variable losses frequently occurred when preparations were kept for a period and frequently frozen and thawed. Data for a typical preparation are shown in Table 1.

*Estimation of protein.* The protein concentration of enzyme solutions was determined by measurement of the optical density at 280 mμ. after appropriate dilution with 0·1 N-NaOH. Under these conditions a solution containing 1 mg. protein/ml. was found to have a density of 1·5.

*Estimation of* pH. This was performed with a pH meter, employing a glass electrode.

## RESULTS

Unfractionated extracts of rabbit-liver–acetone powder showed little increase in the rate of serine synthesis upon addition of pyridoxal phosphate to the otherwise complete reaction medium. After purification of the enzyme by heat treatment and by ammonium sulphate fractionation, however, stimulation of serine synthesis by added pyridoxal phosphate became greater, and at the stage of the ammonium sulphate-fractionated enzyme pyridoxal phosphate usually produced about a twofold increase in the rate of serine synthesis (Table 2). Pre-incubation of the enzyme with pyridoxal phosphate before addition of substrates caused no greater activation than addition of pyridoxal phosphate to the enzyme with the substrates. The

Table 2. *Activation of serine synthesis by pyridoxal phosphate at various stages of enzyme fractionation*

The enzyme was tested under standard conditions. Pyridoxal phosphate when present was added to give a concentration of $5 \times 10^{-4}$ M. Specific activity of the enzyme is expressed as μmoles serine synthesized/hr./mg. protein.

| Enzyme preparation | Specific activity: Without pyridoxal phosphate | Specific activity: With pyridoxal phosphate |
|---|---|---|
| Phosphate extract of acetone powder | 2·73 | 3·78 |
| Supernatant after heat treatment | 4·78 | 7·50 |
| Fractionated with ammonium sulphate | 10·00 | 20·90 |

Table 3. *Effect of various concentrations of pyridoxal phosphate on serine synthesis by ammonium sulphate-fractionated enzyme*

Enzyme purified to first ammonium sulphate fractionation: specific activity 17 μmoles/hr./mg. protein. Tested under standard conditions.

| Concn. of added pyridoxal phosphate (M) | Serine synthesized (μmoles) |
|---|---|
| None | 12·9 |
| $2 \times 10^{-5}$ | 15·2 |
| $10^{-4}$ | 18·5 |
| $5 \times 10^{-4}$ | 26·8 |
| $2·5 \times 10^{-3}$ | 17·4 |

concentration of pyridoxal phosphate required to produce maximum increase of synthesis with such enzyme preparations was rather high, usually about $5 \times 10^{-4}$ M, as may be seen from Table 3.

Enzyme preparations at this or the later stage of purification did not show any increased requirement for added pyridoxal phosphate after dialysis for several days. Although considerable loss in enzymic activity occurred during prolonged dialysis, addition of pyridoxal phosphate to the dialysed preparations did not stimulate serine

synthesis any more than with undialysed preparations. Most other attempts to produce an enzyme preparation with an absolute requirement for pyridoxal phosphate, for example by treating the enzyme with high salt concentrations even at temperatures up to 60°, followed by dialysis, were unsuccessful.

### *Effect of mildly alkaline* pH *on pyridoxal phosphate requirement*

All enzyme preparations were found to undergo considerable inactivation at pH lower than 6 or higher than 8. After keeping the enzyme system at pH 8·6–9·6 and 37° for 30 min. synthetic activity was largely lost, but a large proportion of the activity could be restored to enzyme preparations thus inactivated by subsequently equilibrating the solution with 0·01 N-$NaHCO_3$ under $N_2+CO_2$ (95:5) at 37°, when the pH of the solution was quickly adjusted to 7·2, and then incubating the enzyme with pyridoxal phosphate for a short period before addition of substrate (Table 4). At higher pH values the enzyme was inactivated more completely, but recovery of activity during incubation with pyridoxal phosphate was lower. A trial was carried out with each batch of enzyme to determine the pH at which the enzyme should be incubated in order to obtain subsequently the greatest activating effect with pyridoxal phosphate. This was usually about pH 9.

Table 4. *Effect of incubating the enzyme at alkaline* pH *on requirement for pyridoxal phosphate*

Enzyme was purified to the stage of ammonium sulphate precipitation; specific activity 22 μmoles/hr./mg. protein; 138 units/ml. Four samples of equal volume were diluted with 27·3 vol. water, adjusted with 0·1 N-NaOH to the respective pH values shown and kept at 37° for 30 min. Samples of these solutions corresponding to 0·06 ml. original solution, or 8·3 enzyme units, were used for activity tests. Samples were quickly equilibrated with 0·01 M-$NaHCO_3$ under $N_2+CO_2$ (95:5) at 37° both in the presence and absence of $5\times10^{-4}$ M pyridoxal phosphate, when the solutions rapidly reached pH 7·2. After 30 min. at 37° substrates were added from the side arms and the assay was continued as normally.

| pH of enzyme during 30 min. incubation | Serine synthesized (μmoles): Without pyridoxal phosphate | Serine synthesized (μmoles): With pyridoxal phosphate |
|---|---|---|
| 6·6 | 5·36 | 9·48 |
| 8·6 | 2·01 | 6·12 |
| 9·1 | 0·00 | 4·50 |
| 9·6 | 0·13 | 1·43 |

Some experiments described below were performed with undialysed solutions of the ammonium sulphate-precipitated enzyme, but for most experiments ethanol-precipitated preparations were used. Unless otherwise stated, experiments referred to in tables and figures were performed with enzyme of specific activity 15–30 μmoles serine/hr./mg. protein which had been purified by the complete procedure. Some work was performed with preparations which had been stored some time or which had been frequently frozen and thawed, and which possessed specific activity 2–10 μmoles serine/hr./mg. protein. Enzyme preparations at the ammonium sulphate stage were found to behave in the same manner as ethanol-precipitated enzyme,

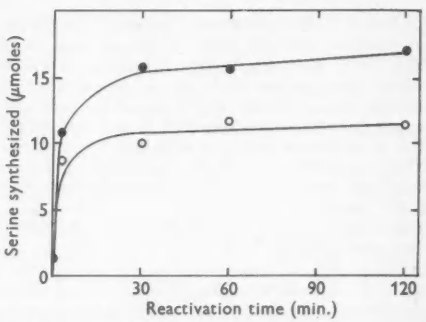


Fig. 2. Effect of incubating inactivated enzyme with pyridoxal phosphate for varying periods of time. Enzyme, 33·2 units/flask, was inactivated by holding at pH 9·1 and 37° for 30 min., then incubated at pH 7·2 and 37° with $2\times10^{-4}$ M pyridoxal phosphate, ●; or with $10^{-5}$ M pyridoxal phosphate, ○, before adding substrates. Serine synthesis determined under standard conditions.

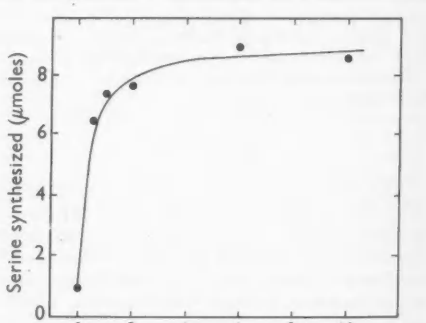


Fig. 3. Effect of incubating inactivated enzyme with pyridoxal phosphate of various concentrations. Enzyme, 18·5 units/flask, was inactivated at pH 9·1 and 37° for 30 min., then incubated at pH 7·2 and 37° with pyridoxal phosphate of various concentrations for 30 min. before adding substrates and measuring serine synthesis under standard conditions.

and no difference in behaviour was observed in preparations partly inactivated during storage or by freezing and thawing.

Reactivation by pyridoxal phosphate of enzyme treated at mildly alkaline pH was very rapid. Thus 70% of the maximum activity was obtained when the substrates were added to the enzyme only a few minutes after the addition of pyridoxal phosphate (Fig. 2). If the enzyme was incubated with pyridoxal phosphate for longer periods before addition of substrates, slightly greater activity was obtained, the maximum reactivation being achieved by pre-incubating the enzyme with pyridoxal phosphate for about 2 hr. (Fig. 2).

The concentration of pyridoxal phosphate required to produce maximum reactivation of the enzyme was quite low. Thus it may be seen by interpolation in Fig. 3 that incubation of the enzyme with $10^{-6}$M pyridoxal phosphate produced about half the maximum rate of synthesis.

*Effect of pyridoxal phosphate on serine breakdown*

Since the enzyme system catalyses both serine synthesis and breakdown, addition of pyridoxal phosphate to enzyme inactivated at pH 9 should stimulate serine breakdown. In preliminary experiments designed to test this, serine breakdown was followed by measuring formaldehyde production, but beyond providing a qualitative demonstration of pyridoxal phosphate activation of serine breakdown the results were unsatisfactory. Recovery of formaldehyde was erratic, and tetrahydroPGA and pyridoxal phosphate interfere with its colorimetric determination. Moreover, the equilibrium appears to favour synthesis, so that only a small proportion of the serine added is converted into glycine and formaldehyde. Determination of serine disappearance gave more consistent results.

Portions of enzyme inactivated by incubation for 30 min. at pH 9·2 and 37° were placed in Warburg vessels. Sodium bicarbonate and tetrahydroPGA were added so that their final concentration was 0·01 and 0·0013M respectively, and water was added so that the final volume on addition of serine was 3 ml. After equilibrating the mixtures with $N_2 + CO_2$ (95:5) (v/v) at 37° for 15 min. in the absence and presence of pyridoxal phosphate respectively, the reaction was started by addition of 25 $\mu$moles L-serine from the side arms of the vessels. The reaction was stopped after 1 hr., and determination of residual serine by the usual method showed that enzyme equilibrated with $5 \times 10^{-5}$M pyridoxal phosphate had catalysed the disappearance of 4·1 $\mu$moles serine, while in absence of pyridoxal phosphate only 0·7 $\mu$mole had disappeared. Residual serine in both cases was compared with a control in which tetrahydroPGA was omitted.

*Specificity of the requirement for pyridoxal phosphate*

Enzyme inactivated at pH 9 has a specific requirement for pyridoxal phosphate (Table 5). Pyridoxamine 5-phosphate, pyridoxine 5-phosphate and deoxypyridoxine 5-phosphate caused negligible reactivation of the enzyme under the same conditions in which pyridoxal phosphate produced its effect.

Table 5. *Specificity of the requirement for pyridoxal phosphate*

Enzyme inactivated for 30 min. at pH 9·1 and 37°; incubated with compound under test for 30 min. at pH 7·2 before addition of substrates. In the first experiment 33·2 units ammonium sulphate-precipitated enzyme, specific activity 22 $\mu$moles/hr./mg. protein, were used per flask. In the second experiment 48·3 units ethanol-precipitated enzyme were used per flask. Activity tested under standard conditions.

| Activator added | Serine synthesized ($\mu$moles) |
|---|---|
| Expt. 1 | |
| None | 1·9 |
| Pyridoxal 5-phosphate ($10^{-4}$M) | 14·4 |
| Pyridoxamine 5-phosphate ($10^{-4}$M) | 3·3 |
| Pyridoxamine 5-phosphate ($5 \times 10^{-4}$M) | 3·5 |
| Expt. 2 | |
| None | 1·9 |
| Pyridoxal 5-phosphate ($5 \times 10^{-5}$M) | 20·7 |
| Pyridoxine 5-phosphate ($2 \times 10^{-4}$M) | 3·1 |
| 2-Deoxypyridoxine 5-phosphate ($2 \times 10^{-4}$M) | 2·8 |

*Inhibition of serine synthesis by pyridoxine phosphate and deoxypyridoxine phosphate*

It may be seen from Fig. 4 that deoxypyridoxine 5-phosphate is a strong inhibitor of the enzyme. If both deoxypyridoxine phosphate and pyridoxal phosphate were incubated together with the inactivated enzyme for 15 min. before the addition of substrates, much less serine synthesis occurred than when the enzyme was incubated with pyridoxal phosphate alone. Thus when pyridoxal phosphate and deoxypyridoxine phosphate, each at a concentration of $5 \times 10^{-5}$M, were incubated with the enzyme before addition of substrates, serine synthesis was inhibited about 35%. If the inactivated enzyme was first incubated with pyridoxal phosphate for 15 min. and then deoxypyridoxine phosphate added and the mixture kept a further 15 min. at 37° before addition of substrates, inhibition was considerably less at all concentrations of deoxypyridoxine phosphate than in the former case. Very marked inhibition was produced if the procedure was reversed, i.e. the inactivated enzyme was incubated for 15 min. with deoxypyridoxine phosphate before the 15 min.

reactivation with pyridoxal phosphate. In this case 67% inhibition resulted when pyridoxal phosphate and deoxypyridoxine phosphate were both added at a concentration of $5 \times 10^{-5}$M. With the last procedure 50% inhibition was produced by about $2 \times 10^{-5}$M deoxypyridoxine phosphate.

Pyridoxine phosphate was a weaker inhibitor of the enzyme than deoxypyridoxine phosphate (Fig. 4). Even when incubated with the enzyme before reactivation by pyridoxal phosphate an inhibitor concentration of $2 \times 10^{-4}$M produced only 25% inhibition.

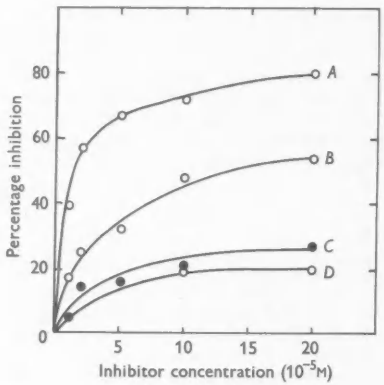


Fig. 4. Inhibition of serine synthesis by deoxypyridoxine phosphate or pyridoxine phosphate. Enzyme, 20–25 units/flask, inactivated by incubating at pH 9·1 and 37° for 30 min. Curve *A*: inactivated enzyme incubated with various concentrations of deoxypyridoxine phosphate for 15 min. at 37° and pH 7·2; pyridoxal phosphate then added and the mixture incubated for a further 15 min. before adding substrates. Curve *B*: inactivated enzyme incubated with both pyridoxal phosphate and various concentrations of deoxypyridoxine phosphate for 15 min. at 37° and pH 7·2 before adding substrates. Curve *C* (●): as for curve *A*, but pyridoxine phosphate used instead of deoxypyridoxine phosphate. Curve *D*: inactivated enzyme incubated with pyridoxal phosphate for 15 min. at 37° and pH 7·2; various concentrations of deoxypyridoxine phosphate then added and the mixture incubated a further 15 min. before adding substrates. Activity tested under standard conditions in all cases. Pyridoxal phosphate concentration $5 \times 10^{-5}$M in all cases.

### *Effect of cations on serine synthesis*

In previous studies none of the bivalent cations tested was found to stimulate serine–glycine interconversion, and ethylenediaminetetraacetate (0·01M) did not inhibit the reaction (Blakley, 1954*d*). This was interpreted as evidence against the participation of multivalent cations in the reaction. Studies on a non-enzymic cleavage of serine to glycine and formaldehyde with pyridoxal as catalyst have demonstrated, however, that in this non-enzymic reaction the presence of certain cations is essential (Metzler, Longenecker & Snell, 1954). On this and other evidence a general mechanism has been proposed for the action of pyridoxal and related compounds in both enzymic and non-enzymic reactions (Metzler, Ikawa & Snell, 1954), and in the proposed scheme the chelation of a cation plays an important part.

In view of the discrepancy between results with the enzymic and non-enzymic systems respectively, the effect of cations on the partially purified enzyme system has been investigated. The results, shown in Table 6, are in agreement with previous findings, for, while they demonstrate inhibition by many cations in varying degree, none of the cations tested was an activator of enzymic serine synthesis either with the normal or reactivated enzyme. Among cations showing an inhibitory effect, $Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$ and $Ni^{2+}$ showed particularly strong effects. Ethylenediaminetetraacetate (0·01M) was again found to produce no inhibition of serine synthesis either with normal or reactivated enzyme.

Table 6. *Effect of cations on the enzymic synthesis of serine*

Serine synthesis measured under standard conditions either with purified enzyme, 10–20 units/flask, in the presence of $5 \times 10^{-4}$M pyridoxal phosphate or with the same enzyme after inactivation at pH 9, 37° for 15 min. and reactivation with $5 \times 10^{-5}$M pyridoxal phosphate at 37°, pH 7·2 for 15 min. Metals added as chlorides except $Fe^{2+}$, $Fe^{3+}$, $Cu^{2+}$, $Ni^{2+}$ and $Al^{3+}$, which were added as sulphates, and $Zn^{2+}$, which was added as acetate.

| Cation added (0·001M) | Inhibition (%) Normal enzyme | Reactivated enzyme |
|---|---|---|
| $Cu^{2+}$ | 96 | 95 |
| $Zn^{2+}$ | 87 | 84 |
| $Ni^{2+}$ | 99 | 73 |
| $Mn^{2+}$ | 39 | 78 |
| $Cd^{2+}$ | 71 | 39 |
| $Co^{2+}$ | 61 | 38 |
| $Fe^{2+}$ | 62 | 19 |
| $Fe^{3+}$ | 41 | 2 |
| $Al^{3+}$ | 33 | 0 |
| $Mg^{2+}$ | 9 | 16 |
| $Ca^{2+}$ | 8 | 0 |

### *Spectrophotometric evidence for Schiff's-base formation*

Schiff's-base formation between pyridoxal phosphate and glycine is generally assumed to be the first step in serine synthesis. It was noticed during this work that addition of glycine to a solution of pyridoxal phosphate in bicarbonate buffer causes

a marked and rapid increase in the intensity of the yellow colour of the solution. Determination of the absorption spectrum of pyridoxal phosphate in the presence and absence of glycine revealed a marked alteration in the spectrum on addition of glycine, consistent with Schiff's-base formation (Fig. 5). A similar change in the absorption spectrum of a pyridoxal phosphate solution resulted when serine was added instead of glycine, the two absorption maxima of the new spectrum appearing at the same wavelengths and having almost identical extinctions at the same concentration of amino acid. Pyridoxal phosphate alone had a single band with maximum at 388 m$\mu$. ($\epsilon_{max.}$ 5700). In the presence of 0·01 M amino acid, maxima appeared at 277 m$\mu$. ($\epsilon_{max.}$ 5000) and 405 m$\mu$. ($\epsilon_{max.}$ 5800); 0·1 M amino acid produced maxima at 279 m$\mu$. ($\epsilon_{max.}$ 5700) and 413 m$\mu$. ($\epsilon_{max.}$ 6600). Addition of glycine to a solution of pyridoxamine phosphate did not produce such a change in spectrum.

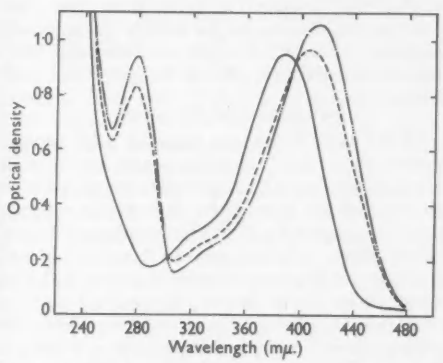


Fig. 5. Absorption spectra of pyridoxal phosphate ($1{\cdot}67 \times 10^{-4}$ M) in $NaHCO_3$–$CO_2$ buffer ($NaHCO_3$ 0·01 M, equilibrated with $N_2+CO_2$ (95:5), pH 7·2). ——, Alone; – – –, in the presence of 0·01 M glycine, (adjusted to pH 7·2); –·–·–, in the presence of 0·10 M glycine (adjusted to pH 7·2).

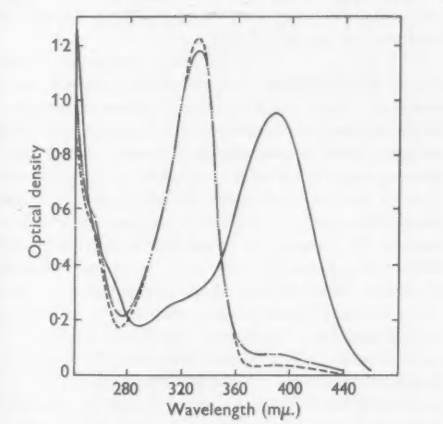


Fig. 6. Absorption spectra of pyridoxal phosphate ($1{\cdot}67 \times 10^{-4}$ M) in $NaHCO_3$ buffer (0·01 M, equilibrated with $N_2+CO_2$ (95:5), pH 7·2). ——, Alone; – – –, in the presence of 0·0067 M-$NaHSO_3$; –·–·–, in the presence of 0·0067 M-$NaHSO_3$ and 0·01 M glycine (adjusted to pH 7·2).

When bisulphite was added to a pyridoxal phosphate solution the solution became colourless, the single absorption band shifting to 330 m$\mu$. ($\epsilon_{max.}$ 7400) (Fig. 6). Addition of 0·01 M glycine to this solution caused only minor changes in the absorption spectrum (Fig. 6), providing further evidence that it was the Schiff's base which was formed between glycine and pyridoxal phosphate in the absence of bisulphite.

## DISCUSSION

Pyridoxal phosphate has been shown to stimulate the synthesis of serine by enzyme preparations from rabbit-liver–acetone powder, the activating effect of pyridoxal phosphate becoming marked after ammonium sulphate fractionation of the enzyme (Table 2). The maximum effect of pyridoxal phosphate was obtained at a concentration of $5 \times 10^{-4}$ M, which caused a doubling of the rate of serine synthesis (Table 3).

When the enzyme was maintained at about pH 9 and a temperature of 37° for 15–30 min. before testing its activity, the rate of serine synthesis was found to fall to a very low value. Such enzyme preparations were markedly reactivated by short incubation with pyridoxal phosphate before addition of substrates, 50% or more of the activity being recovered (Table 4). For this reactivation process pyridoxal phosphate produced half its maximum effect at about $2 \times 10^{-6}$ M (Fig. 3). Investigation of the effect of pyridoxal phosphate on the breakdown of serine presents technical difficulties, but a stimulation of serine disappearance by added pyridoxal phosphate has been demonstrated.

These results are in good agreement with the observation that liver extracts from pyridoxine-deficient pigeons show a reduced ability to synthesize serine from formate (Deodhar & Sakami, 1953), and with the evidence that pyridoxal phosphate, together with PGA or a derivative, is essential for serine synthesis in *Streptococcus faecalis* (Lascelles & Woods, 1954).

The very slight activation of the unfractionated enzyme by added pyridoxal phosphate is most easily explained by assuming that the unfractionated enzyme possesses strongly bound pyridoxal

phosphate. Although no direct evidence has been adduced for this hypothesis, it is in agreement with all the results, and this has been shown for bacterial and mammalian transaminase (O'Kane & Gunsalus, 1947; Schlenk & Snell, 1945; Lichstein, Gunsalus & Umbreit, 1945). As with pig-heart transaminase (O'Kane & Gunsalus, 1947), fractionation of the serine-synthesizing enzyme system with ammonium sulphate increases the activation produced by added pyridoxal phosphate, although in these circumstances the activation by pyridoxal phosphate was not found to cause an increase in serine synthesis appreciably in excess of 100%.

The loss of enzymic activity at pH 9 and concomitant reactivation by added pyridoxal phosphate is presumably due to a modification, at mildly alkaline pH, of the linkages by which pyridoxal phosphate is bound by the enzyme. Even without removing the pyridoxal phosphate, presumed to have been released from the enzyme by treatment at pH 9, the enzymic activity was reduced to a very low figure in the absence of added pyridoxal phosphate. The enzyme cannot therefore recapture any but a small fraction of the released pyridoxal phosphate, even when the solution is again adjusted to pH 7·2. This is probably due to non-specific adsorption of the released pyridoxal phosphate by inert protein present in the relatively impure enzyme preparations used so far. It is noteworthy, however, that after the enzyme had been inactivated at pH 9·0 pyridoxal phosphate at a concentration as low as $2 \times 10^{-6}$ M produced 50% of the maximum reactivation, indicating a high affinity of the enzyme for free pyridoxal phosphate.

It was desirable to test whether pyridoxal phosphate added to the inactivated enzyme became strongly bound at neutral pH. Experiments designed to test this point, in which enzyme reactivated with added pyridoxal phosphate was dialysed before its synthetic activity was tested in the presence and absence of added pyridoxal phosphate, were inconclusive. Large irreversible losses of enzymic activity occurred during dialysis and these obscured a possible loss of activity due to removal of free pyridoxal phosphate.

Serine synthesis by the reactivated enzyme was inhibited by deoxypyridoxine phosphate and pyridoxine phosphate in a manner parallel in many respects to the inhibition of transaminase by these compounds (Meister, Sober & Peterson, 1954). Thus deoxypyridoxine phosphate inhibits serine synthesis most strongly when the enzyme is incubated with the inhibitor before reactivation of the enzyme by pyridoxal phosphate, less markedly when the pyridoxal phosphate and inhibitor are added simultaneously to the enzyme, and only slightly when the enzyme is reactivated by pyridoxal phosphate before addition of inhibitor (Fig. 4).

The serine-synthesizing enzyme and transaminase appear to differ in their relative affinities for pyridoxal phosphate, deoxypyridoxine phosphate and pyridoxine phosphate respectively. Thus the serine-synthesizing enzyme is inhibited to a much greater extent by deoxypyridoxine phosphate than by pyridoxine phosphate (Fig. 4), whereas transaminase is inhibited similarly by these compounds (Meister *et al.* 1954). The affinity of the serine-synthesizing enzyme for deoxypyridoxine phosphate is very similar to its affinity for pyridoxal phosphate (Fig. 4), whereas transaminase has a considerably stronger affinity for pyridoxal phosphate.

*Mechanism of the reaction*

Schiff's-base formation between the 4-formyl group of pyridoxal phosphate and the α-amino group of amino acids is generally supposed to be involved in the mechanism of enzymic reactions catalysed by pyridoxal phosphate (Metzler & Snell, 1952). Direct evidence for the formation of such a compound between glycine or serine and pyridoxal phosphate in neutral aqueous solution has been provided by spectrophotometric data. The addition of 0·01 M glycine or serine to a solution of pyridoxal phosphate ($1{\cdot}67 \times 10^{-4}$ M) in bicarbonate buffer pH 7·2 caused a shift in the absorption band from 388 to 413 mμ. (Fig. 5). At the same time a new absorption band appeared with maximum at 279 mμ. A similar and somewhat greater effect was produced by addition of 0·10 M glycine or serine to the pyridoxal phosphate solution, but the change in spectrum was very largely complete at an amino acid concentration of 0·01 M.

The change in the absorption spectrum of pyridoxal phosphate in the presence of glycine is similar to that which occurs in the spectrum of salicylaldehyde on addition of ethylenediamine, where a shift of an absorption band from 324 to 408 mμ. occurs (Kiss & Auer, 1941). In both cases it may be presumed that Schiff's-base formation causes the shift of the absorption band towards the visible by providing a double bond conjugated with the unsaturated ring system. Further evidence that glycine and pyridoxal phosphate form a Schiff's base was provided by the fact that bisulphite, by suppressing resonance between the aldehyde group and the ring, shifted the absorption band of pyridoxal phosphate from 388 to 330 mμ., and that in the presence of 0·0067 M bisulphite 0·01 M glycine produced no further change in the spectrum of pyridoxal phosphate (Fig. 6).

In recent discussions of the mechanism of pyridoxal phosphate-catalysed enzyme reactions it has been proposed that Schiff's bases formed by pyridoxal phosphate and amino acids are stabilized by

chelating a multivalent cation (Baddiley, 1952; Metzler, Ikawa & Snell, 1954), the chelate ring maintaining planarity of the conjugate ring system. In addition, it has been suggested that the chelated metal ion acts by providing an additional electron-attracting group that operates in the same direction as the nitrogen atom of the pyridyl ring, thus increasing electron displacements from the α-carbon atom of the amino acid (Metzler, Ikawa & Snell, 1954). On the other hand, enzymic synthesis of serine was not stimulated by any of the cations tested with either normal or reactivated enzyme. $Ca^{2+}$ and $Mg^{2+}$ had no significant effect; $Co^{2+}$, $Fe^{2+}$, $Al^{3+}$ and $Fe^{3+}$ were slightly inhibitory; and $Zn^{2+}$, $Mn^{2+}$, $Cu^{2+}$, $Cd^{2+}$ and $Ni^{2+}$ produced marked inhibition (Table 6). Ethylenediaminetetraacetate (0·01 M) caused no inhibition. Similarly, purified transaminase appears not to require cations for activity (Meister *et al.* 1954).

The view that chelation of a metal ion is essential to enzymic reactions involving pyridoxal phosphate is perhaps tenable, despite failure of added cations to stimulate serine synthesis, on the assumption that a metal is an integral part of the enzyme. Evidence for this must await further purification of the enzyme. Alternatively, it is conceivable that the binding of pyridoxal phosphate and the amino acid to the enzyme surface is sufficient to maintain planarity of the system, and that interaction of the carboxyl group, phenolic hydroxyl, amino nitrogen atom and pyridyl nitrogen respectively with groups on the enzyme surface is even more favourable than chelation of a cation to the requisite electromeric displacement of electrons.

## SUMMARY

1. A manometric method is presented for the estimation of the activity of the enzyme system synthesizing serine from glycine and formaldehyde.

2. The enzyme system has been partially purified by heat treatment, ammonium sulphate fractionation and ethanol fractionation.

3. During enzyme fractionation the requirement for pyridoxal phosphate increased. A twofold increase in the activity of the partially purified enzyme was produced by addition of $5 \times 10^{-4}$ M pyridoxal phosphate to the reaction mixture.

4. Enzyme incubated at about pH 9 and 37° for 15–30 min. was largely inactivated, but a substantial proportion of the activity could be recovered by incubating the enzyme with pyridoxal phosphate at pH 7·2 for a short period.

5. Half the maximum reactivation was produced by $10^{-6}$ M pyridoxal phosphate. Pyridoxamine phosphate, pyridoxine phosphate and deoxypyridoxine phosphate produced negligible reactivation of the enzyme.

6. Deoxypyridoxine phosphate and, to a lesser extent, pyridoxine phosphate inhibited the reactivated enzyme, particularly when added to the inactivated enzyme before its reactivation with pyridoxal phosphate.

7. Ethylenediaminetetraacetate (0·01 M) did not inhibit serine synthesis by either normal or reactivated enzyme. Bi- and ter-valent cations did not stimulate serine synthesis; on the contrary, many cations were found to be strong inhibitors.

8. Spectrophotometric evidence is presented for the formation of Schiff's bases between pyridoxal phosphate and amino acids in neutral aqueous solution. These results are discussed in relationship to proposed mechanisms of enzymic reactions involving pyridoxal phosphate.

I am greatly indebted to Dr A. L. Morrison of Roche Products Ltd. for generous supplies of crystalline pyridoxal phosphate, and to Dr H. A. Sober for crystalline samples of the other pyridoxine derivatives used. I wish to thank Professor A. H. Ennor for helpful advice and criticism, and Mr T. Curtis for technical assistance.

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# Electrophoretic Separation and Determination of Flavins

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The available chemical, biological and microbiological methods for the determination of individual flavins of biological interest [flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN, riboflavin 5′-phosphate), riboflavin] and of their commonest breakdown products (lumichrome, lumiflavin) have proved unsatisfactory when applied to a mixture of these compounds.

Attempts to separate different flavins in a mixture have been made by previous authors (Crammer, 1948; Hais & Pécakova, 1949; Carter, 1950; Yagi, 1951; Dimant, Sanadi & Huennekens, 1952; Huennekens, Sanadi, Dimant & Schepartz, 1953), using paper chromatography. They did not obtain, however, a complete resolution of all flavins, nor could they determine quantitatively the separated compounds. Siliprandi, Siliprandi & Lis (1954) have recently separated riboflavin, FMN and FAD by paper electrophoresis. To our knowledge no convenient procedure has, however, been reported for separating all the flavins of a mixture.

A method for the separation and estimation of all the flavins normally encountered is described in this communication.

## EXPERIMENTAL

*Materials.* Riboflavin was a commercial preparation obtained from H. La Roche. FMN was prepared according to Viscontini, Ebnoether & Karrer (1952), FAD according to Siliprandi & Bianchi (1955), lumiflavin according to Warburg & Christian (1932), and lumichrome according to Karrer, Salomon, Schopp & Schlittler (1934).

### *Electrophoresis of flavins*

*Paper electrophoresis.* A mixture containing not more than 7 $\mu$g. each flavin was applied in one spot to a strip (6 cm. × 25 cm.) of Munktell 20 paper, at a distance of 10 cm. from the cathode. The paper was then moistened with a buffer solution, except where the mixture had been applied, and lightly pressed between two sheets of filter paper to remove excess of liquid. The electrophoresis was run in sodium acetate buffer, pH 5·1, $I = 0{\cdot}05$. A potential of 380 v was applied for 6 hr., giving a current of 1·2 mA/cm. width.

For qualitative electrophoresis the paper was placed between two glass plates firmly clamped together. The ends of the strips were cut off, at the end of the run, in order to prevent the spread of the liquid from the buffer solution into the paper, and the paper was dried in a stream of hot air. The spots were located with a Mineralight short-wave ultra-

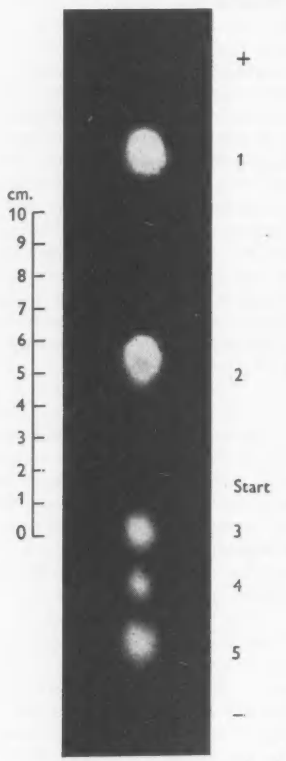


Fig. 1. Separation of a mixture of flavins by paper electrophoresis: sodium acetate buffer, pH 5·1, $I$ 0·05; 1·2 mA/cm. during 6 hr. 1, FAD; 2, FMN; 3, lumiflavin; 4, lumichrome; 5, riboflavin. The photograph was taken in ultraviolet light, with panchromatic plates and a green filter on the objective; exposure time, 45 sec.

violet lamp, a filter SL 2537 (Ultraviolet Products Inc., South Pasadena, California) being used, and their identification was carried out by reference to the spots obtained from a known mixture in a parallel run.

Spots due to riboflavin and FMN show a bright-yellow, and the spot due to FAD shows a pale-yellow, fluorescence. The spot due to lumiflavin shows a greenish yellow, and that due to lumichrome a bright sky-blue, fluorescence.

The separation of the most important flavins is shown in Fig. 1. FAD and FMN migrate towards the anode, FAD being twice as fast as FMN; riboflavin, lumiflavin and lumichrome migrate in decreasing order of mobility towards the cathode.

No breakdown of the pure compounds was observed when these were run separately. For this reason, and because the spots are sharply defined without tailing, the electrophoretic separation can be usefully applied to the quantitative estimation of the different flavins in a mixture.

For this purpose an accurately measured volume of the solution to be analysed was applied on the paper from an Agla micrometer syringe (Burroughs Wellcome Ltd.). The electrophoresis was run with the paper hanging free in a closed chamber; all the other described conditions were maintained with the exception of drying the paper, which was done in a fume cupboard at room temperature. The spots were detected and identified as described above.

Strips of equal size and shape, each containing one spot, were cut out and eluted with water as described by Brimley & Barret (1953), at a flow rate of 1 ml./hr.; in each case 8 ml. eluate were collected in a graduated tube. This volume was usually sufficient to elute the substances completely, but occasionally a larger volume was collected as a check on recovery.

The fluorescence of the eluate was determined in a Coleman 12 B photofluorimeter (Coleman Instruments Inc., Maywood, Illinois), the fluid collected under the same conditions from a similar piece of filter paper from the same strip being used as a blank. The amount of flavin was estimated by comparing the fluorescence with that of a known solution of the pure compound.

However, from the measurement of fluorescence the recovery of riboflavin, FMN and FAD appeared to be incomplete. For this reason the recovery for these flavins was also estimated by measuring the extinction coefficients of the eluates at 260 and 450 m$\mu$. A concentration of flavins suitable for spectrophotometric measurements was obtained by depositing mixtures containing up to 50 $\mu$g. of each of these compounds in a line on the filter paper. Results reported in Table 1 show that when determined spectrophotometrically the recovery of riboflavin FMN and FAD, as well as that of the other flavins, is complete.

*Cellulose-column electrophoresis.* By use of cellulose-column electrophoresis a resolution of larger amounts of flavins was obtained. The apparatus and the technical details were according to Flodin & Porath (1954).

Table 1. *Elution of flavin mixtures separated by paper electrophoresis*

Electrophoresis on Munktell 20 paper, using sodium acetate buffer, pH 5·1, $I = 0{\cdot}05$; potential applied 380 v, giving a current of 1·2 mA/cm., for 6 hr. Eluent, water. Data reported under *A* are calculated from measurements of the fluorescence of the eluates; data under *B* are calculated from measurements of the extinction coefficients at 260 and 450 m$\mu$.

| Substance | Amount applied ($\mu$g.) | Amount recovered ($\mu$g.) | | Recovery (%) | |
|---|---|---|---|---|---|
| | | *A* | *B* | *A* | *B* |
| Riboflavin | 0·5 | 0·24 | — | 48 | — |
| | 2·5 | 1·23 | — | 49·6 | — |
| | 5 | 2·45 | — | 49 | — |
| | 50 | 24·7 | 53·2 | 49·4 | 106·4 |
| FMN | 0·5 | 0·29 | — | 58 | — |
| | 2·5 | 1·44 | — | 57·7 | — |
| | 5 | 2·93 | — | 58·4 | — |
| | 50 | 29·1 | 56·7 | 58·2 | 113·4 |
| FAD | 0·9 | 0·64 | — | 71·1 | — |
| | 1·8 | 1·28 | — | 71·2 | — |
| | 4·5 | 3·17 | — | 70·7 | — |
| | 7·2 | 5·14 | — | 71·4 | — |
| | 50 | 35·3 | 51·4 | 70·6 | 102·8 |
| Lumiflavin | 0·5 | 0·48 | — | 96 | — |
| | 2·5 | 2·44 | — | 97·6 | — |
| | 5 | 48·6 | — | 97·2 | — |
| Lumichrome | 0·5 | 0·47 | — | 94 | — |
| | 2·5 | 2·31 | — | 92·4 | — |
| | 5 | 46·6 | — | 93·2 | — |

Sodium acetate buffer, pH 5·1, $I$ 0·05, or 0·05 M ammonium formate, pH 4·8, was used. A mixture of flavins containing not more than 1·5 mg. each compound in 4 ml. buffer was put on the top of the column (50 cm. × 3 cm.) of cellulose powder (Whatman B quality, standard grade).

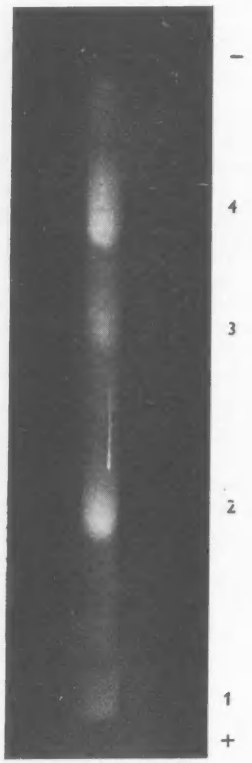


Fig. 2. Electrophoretic separation of a mixture of flavins on a cellulose column (ammonium formate buffer, pH 4·8, 0·05 M; 50 mA for 14 hr.). 1, FMN; 2, riboflavin; 3, lumiflavin, 4, lumichrome. The photograph was taken in ultraviolet light, with panchromatic plates and a green filter on the objective; exposure time: 40 sec.

Before the current was applied the mixture was displaced 15–20 cm. down the column, by allowing a sufficient amount of buffer to flow through the column. The top of the column was then connected to the cathode, the bottom to the anode and a potential of 520 V applied for 14 hr.

The separation of mixtures of flavins by column electrophoresis is shown in Figs. 2 and 3.

On completion of the run the column was disconnected from the electrophoresis apparatus and placed on a fraction collector. The elution was performed with the same buffer as that used for the electrophoresis and the flow rate was adjusted to 25 ml./hr. The effluent was collected in 8 ml. fractions and the extinction coefficients of these solutions were measured in a Beckman model D.U. spectrophotometer at 250 and 450 mμ. The identification of the compounds was carried out with the help of paper electrophoresis as previously described. When necessary, the fractions to be analysed were evaporated under reduced pressure, at 35°, until a suitable concentration for paper electrophoresis was reached. When it was necessary to concentrate the eluate ammonium formate buffer was used, since the constituents are volatile and a smaller amount of buffer ions was left in the concentrate.

Quantitative recoveries have been obtained in these experiments.

*Differential adsorption of flavins on cellulose*

During the preliminary experiments a differential adsorption of some of the flavins on cellulose was noticed. Fig. 4 shows the chromatographic separation of a mixture containing 0·5 mg. FMN, 0·25 mg. riboflavin, 0·1 mg. lumichrome and 0·2 mg. lumiflavin on a column (50 cm. × 3 cm.) packed with cellulose powder (Whatman B quality, standard grade). The same acetate buffer as in the electrophoresis experiments was used as solvent. This procedure, however, does not permit the separation of FAD from FMN.

All the experiments were carried out as far as possible in the absence of light, so as to prevent the breakdown of flavins which are highly photolabile compounds.

## DISCUSSION

The chromatographic procedures reported by previous authors (Crammer, 1948; Hais & Pécakova, 1949; Carter, 1950; Yagi, 1951; Dimant *et al.* 1952; Huennekens *et al.* 1953) for the separation of flavins are not altogether satisfactory. They do not allow a complete resolution of mixtures containing several riboflavin derivatives, the separation of the individual compounds is not sharp, and the $R_F$ values of some of the compounds are rather variable.

The paper-electrophoresis procedure described here permits a sharp and readily reproducible separation of riboflavin and its derivatives, and can therefore be considered as the most suitable technique for the analysis of flavin mixtures in microgram quantities so far proposed. Paper electrophoresis has, moreover, a much higher resolving power than paper chromatography.

Indeed traces of FMN contaminating FAD solutions, and not revealed by paper chromatography, are readily resolved as a distinct spot by paper electrophoresis. Hydrolysis of FAD during the electrophoretic run can be excluded as a reason for this second spot, since no breakdown of flavins has been observed when pure compounds were run separately.

Paper electrophoresis has been successfully applied to the quantitative determination of each component of a flavin mixture. A total recovery has been obtained, although the fluorescence readings on the eluted samples are lower than expected. This may be related to a quenching of the fluorescence during the electrophoresis. A quenching of fluorescence of flavins in saline solu-

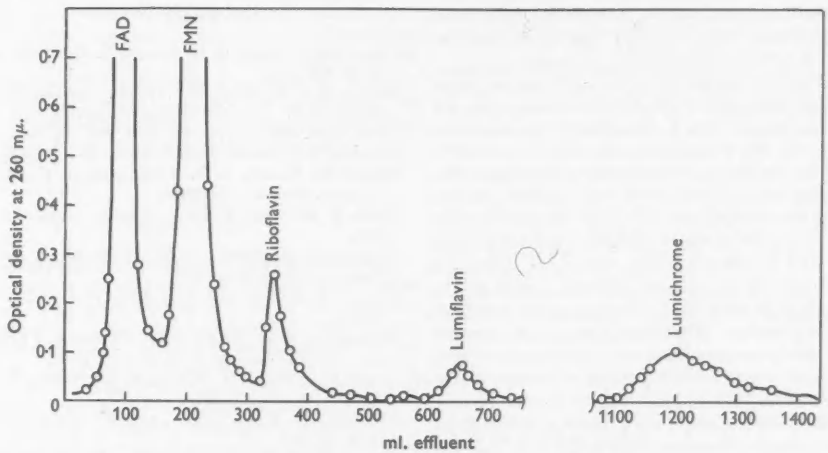


Fig. 3. Elution curves of a mixture of flavins separated by cellulose-column electrophoresis (sodium acetate buffer, pH 5·1, *I* 0·05; 520v for 14 hr.). FAD, 0·3 mg.; FMN, 0·55 mg.; riboflavin, 0·08 mg.; lumiflavin, 0·1 mg.; lumichrome, 0·15 mg.

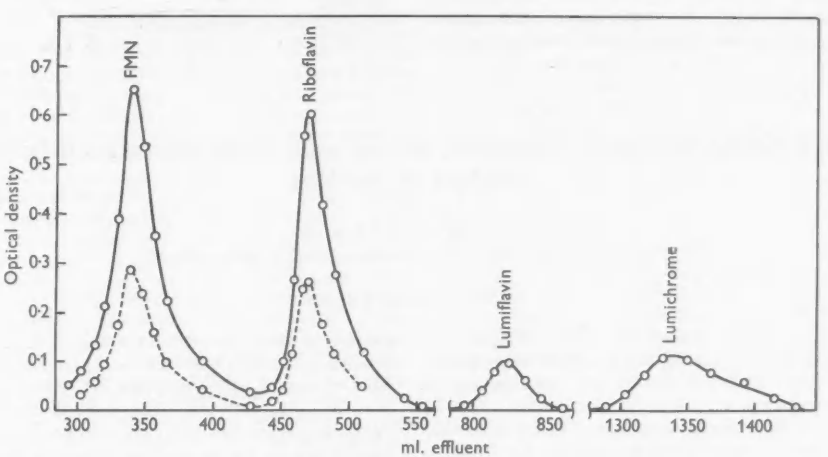


Fig. 4. Elution curves of a mixture of flavins separated by chromatography on cellulose column. Solvent: sodium acetate buffer, pH 5·1, *I* 0·05. Flow rate 30 ml./hr. FMN, 0·5 mg.; riboflavin, 0·25 mg.; lumichrome, 0·12 mg.; lumiflavin, 0·08 mg. —, 260 mμ.; - - - -, 450 mμ.

tions has already been pointed out by Bessey, Lowry & Love (1949) and Weber (1950). However, since the quenching of fluorescence is constant, fluorescence measurements can be used, with an error of $\pm 2\%$, for the determination of the eluted flavins. Fluorimetry has been preferred, as it is 30 times more sensitive than spectrophotometry at 260 m$\mu$. and 100 times more sensitive than colorimetry (Hegsted, 1954).

The separation of larger amounts of each compound has been obtained by using cellulose-column electrophoresis; this method can therefore be employed for micropreparative purposes.

A differential adsorption on cellulose of some flavins has been observed. As will be seen from the elution curves in Fig. 4, the degree of adsorption increases in the following order: FAD (=FMN), riboflavin, lumiflavin, lumichrome. Consequently, when displaced 15 cm. down the column before starting the electrophoresis, the flavin mixture undergoes a partial resolution, its components being distributed in distinct zones. When the current is applied the flavins are at different levels in the column and so start their electrophoretic run from different positions. This fact does not hinder the electrophoretic separation on a cellulose column; however, the distribution pattern of the separated compounds is different from that obtained on paper, where all the substances start from the same place.

The methods described here have been successfully applied to the determination of flavins in biological materials.

## SUMMARY

A method for the separation and quantitative determination of riboflavin and its derivatives (flavin mononucleotide, flavin-adenine dinucleotide, lumichrome and lumiflavin) by paper electrophoresis is described. A micropreparative separation of the same compounds has been achieved by means of cellulose-column electrophoresis.

The work was aided by grants from the Rockefeller Foundation, to which grateful acknowledgement is made. We also wish to thank Mr P. L. Ipata for skilled technical assistance.

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# The Relation between Thiamine, Biotin and Tryptophan Metabolism, Studied in the Rat

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Previous papers (Dalgliesh, 1952; Charconnet-Harding, Dalgliesh & Neuberger, 1953) have shown that useful information on the relationship of B-vitamins to tryptophan metabolism may be obtained by examining urinary metabolites excreted after ingestion of tryptophan by vitamin-deficient animals. The present paper describes a continuation of this work with an elaboration of the technique. The effects of deficiencies of thiamine and biotin have been investigated. Thiamine deficiency has been known for some time to decrease the conversion of tryptophan into nicotinic acid derivatives by the rat (Junqueira & Schweigert, 1948), but there has been no evidence to show at what stage in the conversion thiamine functions. A preliminary survey of other B-vitamins suggested that pantothenic acid, nicotinic acid and biotin had no effect on tryptophan metabolism

in the rat. This agreed with the known lack of effect of pantothenic acid on nicotinic acid formation from tryptophan by the rat (Junqueira & Schweigert, 1948). Moreover, should nicotinic acid, which is derived from tryptophan, be closely linked with tryptophan metabolism, it might be expected that a nicotinic acid deficiency once established should undergo an auto-intensification, which has never been observed. However, Shanmuga Sundaram, Tirunarayanan & Sarma (1954) have recently claimed that biotin is concerned in an early stage of tryptophan metabolism in *Neurospora*. The effect of biotin deficiency on tryptophan metabolism in the rat was therefore reinvestigated under more rigorous conditions. The work described in this paper has been the subject of two preliminary communications (Dalgliesh, 1954, 1955*b*).

## METHODS

*Animals.* White rats, recently weaned, were obtained as required from the stock of the Postgraduate Medical School. These were kept throughout in wire-bottomed metabolism cages to prevent coprophagy. Diet was fed *ad lib.* except when tryptophan was administered. For this purpose, DL-tryptophan (usually approx. 1 mg./g. body wt./day for 1 or 2 days) was mixed in about half the usual amount of diet, in order to encourage complete consumption. The drop in weight, frequently observable in the growth curves after feeding tryptophan, is probably to be attributed to the temporary restriction of food rather than to any effect of the tryptophan *per se* though the palatability may be somewhat reduced. Water was freely available at all times. Acetic acid was added as preservative to the urine receivers.

*Examination of metabolites.* This was carried out by methods reported in detail elsewhere (e.g. Dalgliesh & Tekman, 1953; Dalgliesh, 1955*a*). Briefly, the urine was centrifuged, metabolites in the supernatant were adsorbed on deactivated charcoal, eluted with aqueous phenol, concentrated in a vacuum with simultaneous removal of phenol; the residues were submitted to paper chromatography, using butanol–acetic acid as solvent, and suitable colouring reagents applied (Dalgliesh, 1952, 1955*a*).

*Thiamine-deficient regimes. Diet* 1. Casein (ethanol-extracted for 3 days), 10%; sucrose, 36%; starch, 40%; fat mixture (4 parts arachis oil to 1 part cod liver oil, w/w), 10%; salt mixture (cf. Dalgliesh, 1952), 4%. The following vitamins were added to each kg. diet for the control animals [in the deficient groups the appropriate vitamin(s) were omitted]: thiamine, 10 mg.; riboflavin, 10 mg.; pyridoxine, 10 mg.; nicotinic acid, 10 mg.; *p*-aminobenzoic acid, 25 mg.; Ca pantothenate, 50 mg.; inositol, 100 mg.; folic acid (pteroylglutamic acid), 2 mg.; biotin, 0·1 mg.; vitamin K (Synkavit, Roche Products Ltd.), 1 mg. Each day 50 mg./rat of choline chloride were mixed in the diet as a 5% (w/v) solution. Each rat was individually given daily by dropper a solution of vitamin $B_{12}$ (Cytamen, Glaxo Laboratories Ltd.) equivalent to 0·05 μg./rat/day, and also 0·1 ml./day of a 1% (w/v) solution of α-tocopherol in arachis oil.

*Diet* 2. This was as for Diet 1, except that the amounts/kg. diet of certain vitamins were altered to the following: riboflavin, 40 mg.; nicotinic acid, 100 mg. Choline was decreased to 5 mg./rat/day.

*Diet* 3. This was as for Diet 1, except that B-vitamins were given in solution by stomach tube instead of being mixed in the diet. Stock solutions were made up as follows: thiamine, 45 mg./15 ml. 0·01 N-HCl; pyridoxine hydrochloride, 36 mg./15 ml. 0·01 N-HCl; biotin, 1 mg./10 ml. 50% (v/v) ethanol; Ca pantothenate, 326 mg./100 ml. water; *p*-aminobenzoic acid, 1 g./200 ml. 5% (v/v) ethanol; a mixed solution of 1 g. inositol, 1 g. nicotinic acid and 3 g. choline chloride in 200 ml. 0·1 N-HCl; folic acid, 5 mg./100 ml. 50% (v/v) ethanol. The vitamin mixture for administration by stomach tube was made by adding 20 mg. riboflavin to a mixture of stock solutions as follows: *p*-aminobenzoic acid, 50 ml.; inositol–nicotinic acid–choline, 50 ml.; thiamine, 1·6 ml.; pyridoxine, 2·8 ml.; pantothenic acid, 16·6 ml.; biotin, 1 ml.; folic acid, 20 ml. The mixture was then made up to 500 ml. with water and stored in the cold and dark; 1 ml./day was given to each rat. Vitamin K (Synkavit) and α-tocopherol were given in arachis oil from a dropper (0·1 ml./rat/day containing 1 mg. α-tocopherol and 1 μg. Synkavit). Appropriate vitamin(s) were omitted from the deficient groups.

*Biotin-deficient regime.* The ethanol-extracted casein of Diet 3 was replaced by an equal weight of dried egg albumin (G. T. Gurr Ltd., London, S.W. 6). To the solid diet, before mixing to a thick paste with water, was added 1% (w/w) succinylsulphathiazole. The vitamin mixtures were the same as in Diet 3, except that the quantity of folic acid was doubled, and appropriate vitamin(s) were omitted from the deficient groups.

## RESULTS

During this work various basal vitamin mixtures have been tried, and two modes of vitamin administration compared. In previous work (Dalgliesh, 1952; Charconnet-Harding *et al.* 1953) vitamins have been mixed in the diet. It was suggested to the author by Dr A. M. Copping that more satisfactory results were obtainable if vitamins were given daily in solution by stomach tube. The amount of vitamin consumed is then known with more certainty and, as no allowance need be made for incomplete consumption of food, there is useful economy. The comparisons made in this work have convinced the author that there are considerable advantages in Dr Copping's technique.

Five experiments were carried out in the thiamine-deficiency investigations. All gave the same results, described below, from the point of view of tryptophan metabolism, but growth curves for only two of these experiments are reproduced. Fig. 1 shows curves obtained in an experiment with Diet 1, and illustrates a hazard sometimes encountered when vitamins are mixed in the diet. It will be seen that, after 25 days, growth of the control animals markedly deteriorated. Satisfactory growth of control animals had previously

been obtained on a similar diet, and the work of Kandutsch & Baumann (1953) and Rombouts (1953) on the decomposition of thiamine in laboratory diets makes it probable that the poor growth of the control rats shown in Fig. 1 was due to a thiamine deficiency. In case a vitamin imbalance was involved, another group of animals were given Diet 2, in which the proportions of the vitamins were altered. Growth of the control animals was now comparable to that found for black-and-white rats by Copping, Crowe & Pond (1951), who gave their vitamins by stomach tube, but the net consumption of vitamins on Diet 2 was much higher than in the experiments of Copping *et al.* It seemed likely that thiamine decomposition and possible similar complications would be less likely to arise when using Dr Copping's technique, described under Diet 3. Growth curves obtained on such a regime are shown in Fig. 2. Growth of the control animals was more satisfactory than when the vitamins were mixed in the diet; moreover, once practice had been obtained, administration of vitamins by stomach tube was quicker and less troublesome, the animals easily becoming accustomed to the procedure. Control animals on regimes having the vitamins mixed in the diet showed a higher excretion of tryptophan metabolites after supplementary tryptophan than did animals on a stock diet or those receiving their vitamins by stomach tube, suggesting that in the former case the vitamin intake was less balanced.

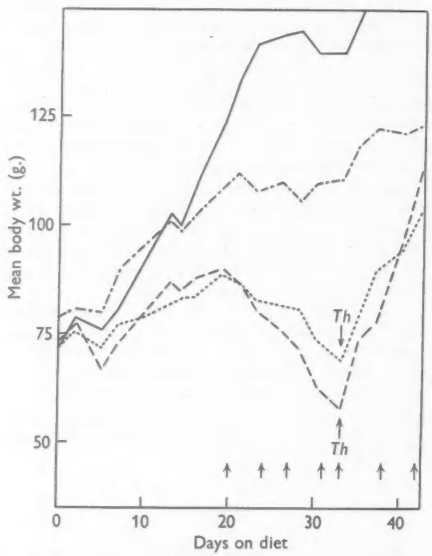



Fig. 1. Growth curves of control (——), thiamine-deficient (– – –), pyridoxine-deficient (·–·–·) and thiamine–pyridoxine-deficient (·····) female rats fed by the vitamins-in-the-diet technique. Each curve is the mean for three animals. Thiamine was given to the deficient animals from the points marked *Th*. Small arrows at the bottom represent supplementary tryptophan in the diet.

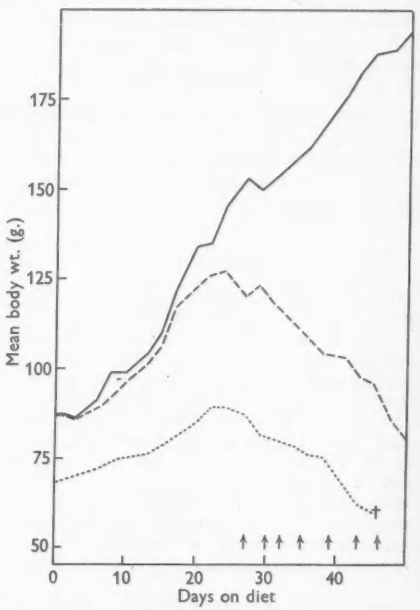



Fig. 2. Growth curves of control (——), thiamine-deficient (– – –) and thiamine–pyridoxine-deficient (·····) female rats given vitamins by the stomach-tube technique. Each curve is the mean for five animals. Small arrows at the bottom represent supplementary tryptophan in the diet. Animals had to be killed at the point marked †.

The results of feeding tryptophan to the various groups of animals were the same in all experiments. At the tryptophan dose level used, the control animals normally excreted small but detectable quantities of tryptophan metabolites. Tryptophan administration was generally started about 3 weeks after initiation of the dietary regime. At this stage excretion by the thiamine-deficient and control rats was indistinguishable, and the rats deficient in thiamine and pyridoxine showed a typical pyridoxine-deficient excretory pattern. As thiamine deficiency became established, as shown by the growth curves, the small excretion of tryptophan metabolites by the tryptophan-fed thiamine-deficient rats ceased. Simultaneously, in the rats

deficient in thiamine and pyridoxine, excretion of the metabolites typical of pyridoxine deficiency decreased and, by the time the animals approached their starting weights, metabolite excretion was confined to a relatively small output of xanthurenic acid. However, kynurenine fed at this stage resulted in a large output of metabolites typical of pyridoxine deficiency. There was no evidence of formylkynurenine excretion on giving tryptophan to animals with a simple thiamine deficiency. On giving thiamine to deficient animals there was a dramatic growth response, as shown in Fig. 1. Simultaneously, the typical pyridoxine-deficient excretory pattern returned in those animals previously doubly deficient.

Typical excretory patterns after establishing both thiamine and pyridoxine deficiencies are summarized in Table 1. It should be emphasized that the metabolites under discussion in this paper are those related to transformation of tryptophan into nicotinic acid. All animals, deficient or otherwise, described in this paper excreted after tryptophan administration appreciable amounts of indolic compounds. The major ones were tryptophan, indican, and two spots moving fast in butanol–acetic acid, the slower moving of which is considered to be principally indole-$\beta$-aceturic acid and the faster moving indoleacetic acid, together with indole derived from faecal contamination of the urine. In the biotin-deficiency experiments, in which succinylsulphathiazole was fed, the indole-aceturic acid spot was smaller than in the other experiments. The indole–indoleacetic acid spot was less markedly decreased.

Several diets were tried in the biotin-deficiency experiments. Biotin-deficient casein-based diets with added aureomycin (vitamins mixed in the diet) gave a slight deficiency as judged by growth curves, but the degree of deficiency was considered quite inadequate for the investigation. Diets with egg albumin (containing the biotin antagonist avidin) as protein source were tried by both the vitamins-in-the-diet and vitamins-by-stomach-tube techniques. Somewhat greater deficiencies were obtained, but again these were considered inadequate. By combining supplementary succinylsulphathiazole with a diet based on egg albumin, marked deficiencies were obtained readily. These results contrast with those of Emerson & Wurtz (1944), who found an egg-albumin diet to be adequate to produce a high degree of biotin deficiency, which was not increased by succinylsulphathiazole. The amounts of succinylsulphathiazole metabolites excreted were not sufficient to interfere with chromatography of the tryptophan metabolites.

Growth curves obtained with the combined egg albumin–succinylsulphathiazole regime are shown in Fig. 3. The biotin-deficient group showed appreciable deficiency (thinning of hair, scale formation) after 4 weeks. Scaly dermatitis, 'spectacle eyes' and 'kangaroo gait' were apparent by the sixth week and marked after the eighth week. The biotin-deficient rats had lost

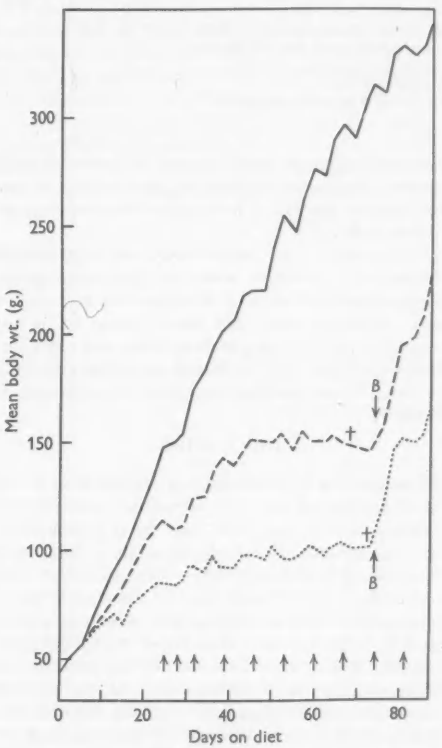



Fig. 3. Growth curves of control (——), biotin-deficient (– – –), and biotin–pyridoxine-deficient (.....) male rats fed on vitamins by the stomach-tube technique. Each curve is the mean for three animals. Small arrows at the bottom represent supplementary tryptophan in the diet. An animal died at the points marked †. Biotin was given to the deficient animals from the points marked *B*.

almost all their hair by the tenth week and at about this stage some oedema became evident. In the group deficient in biotin and pyridoxine the loss of hair was less marked, but the dermatitis was more severe and the eyes became completely closed. The control group, differing from the biotin-deficient group only in receiving biotin, grew well throughout, and the picture was therefore not complicated by any accompanying folic acid deficiency.

Table 1. *Typical metabolite-excretory patterns after feeding tryptophan to control and vitamin-deficient rats*

Excretion represented by +; metabolites sometimes excreted by ±.

| Metabolite | Non-deficient | Thiamine-deficient | Pyridoxine-deficient | Thiamine–pyridoxine-deficient | Biotin-deficient | Biotin–pyridoxine-deficient |
|---|---|---|---|---|---|---|
| Kynurenine | ± | − | + + | ± | ± | + + |
| Kynurenine conjugates | − | − | + + | ± | − | + + |
| Hydroxykynurenine | − | − | + + | ± | − | + + |
| Hydroxykynurenine conjugates | − | − | + + + | ± | − | + + + |
| Xanthurenic acid (4:8-dihydroxy-quinoline-2-carboxylic acid) | + | − | + + + + | + | + | + + + + |
| Anthranilic acid | ± | − | − | − | ± | − |
| Anthranilic acid conjugates | ± | − | − | − | ± | − |

Administration of biotin to the deficient animals caused a dramatic response in growth (Fig. 3) and a noticeable growth of hair in the denuded animals within 24 hr.

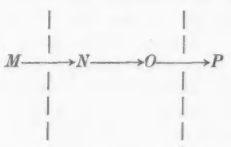
Throughout the experiment no appreciable differences could be seen in the tryptophan-metabolite excretion of biotin-deficient and control rats, and at no stage did there appear to be any change in the typical pyridoxin-deficient excretory pattern of the group deficient in biotin and pyridoxine. These results are also summarized in Table 1.

## DISCUSSION

Investigations on intermediary metabolism in the intact animal can have the advantage over enzymic techniques that reactions may be revealed which are brought about by enzymes too unstable to lend themselves readily to *in vitro* investigations, and that reaction chains may be deduced from the occurrence of some intermediate which could not readily be deduced in an isolated or reconstituted system. Moreover, even if the amount and activity of an enzyme in a given organ are known, its importance in the overall picture may be difficult to assess, other than by a study of end products, as the available enzyme cannot be fully efficient unless provided with the right precursor in the right place at the right time. *In vitro* and *in vivo* experiments are thus complementary, and the remarkable recent advances in enzymic techniques do not reduce the need for experiments on the intact animal.

The approach used in these experiments is analogous for mammals to the study of deficient mutants in micro-organisms. It depends on the function of many B-vitamins as constituents of coenzymes. In a deficiency of such a vitamin an enzymic reaction for which it provides a coenzyme may be inhibited. If one reaction in a metabolic chain is inhibited and a substance prior to it in the chain is administered to the animal, it is to be expected that the substrate of the inhibited reaction (and/or simple derivatives) may be excreted. Such an approach has led to useful information on the function of pyridoxine (Dalgliesh, 1952) and riboflavin (Charconnet-Harding *et al.* 1953) in tryptophan metabolism. The method, of course, has limitations. Thus if a vitamin deficiency results in a definite change in a pattern of metabolite excretion, definite conclusions may be drawn. But if there is deficiency, as shown perhaps by the growth curve, but no change in the pattern of metabolite excretion, it cannot be concluded that that vitamin is not concerned in that metabolic chain. The coenzyme in which the vitamin participates may be concerned in many reactions involving enzymes which have widely differing affinities for the coenzyme. In a deficiency, these reactions are not necessarily inhibited to the same degree. For example, pyridoxal phosphate is the coenzyme in transamination reactions among many others, but transamination can be apparently unaffected by a degree of vitamin $B_6$-deficiency which would result in marked inhibition of other vitamin $B_6$-dependent enzymes (cf. Snell, 1953).

An improved technique is described in this paper which is useful in certain cases. If we have a metabolic chain and a block occurs at stage *b*, then




excretion of *O* after administration of *M* has obvious significance. But it is not necessarily justifiable to deduce from an absence of metabolite excretion that there is a block at *a*. However, if blocks at *a* and *b* are combined, formation of *O* should be inhibited by block *a*, so that the excretion of *O* previously observed with a simple block at *b* should no longer occur. Thus the reality of a block at *a* can be checked.

The general outline of the pathway for conversion of tryptophan into nicotinic acid is summarized in Fig. 4 (for collected evidence see review by Dalgliesh, 1955*c*). Pyridoxine is concerned at the stage marked *C* ... *C* (Dalgliesh, 1952) and riboflavin probably at the stage marked *B* ... *B* (Charconnet-Harding *et al.* 1953). The present results show that thiamine deficiency inhibits formation of the substrate of the pyridoxine-dependent reaction. It must therefore function in the conversion of tryptophan into formylkynurenine, stage *A* ... *A* in the diagram, or in the formation of kynurenine from formylkynurenine. The latter is inherently less probable and is excluded by the absence of formylkynurenine excretion by the thiamine-deficient animals.

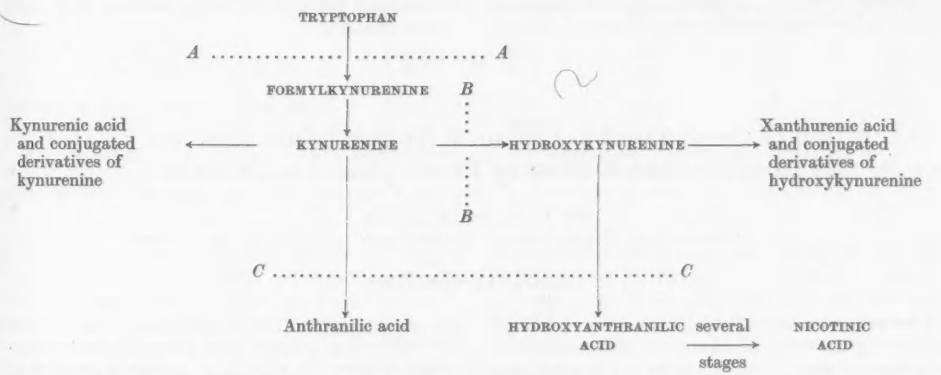



Fig. 4. Outline of the pathway for conversion of tryptophan into nicotinic acid. Intermediates on the main pathway in small capitals, by-products in ordinary type.

These experiments thus establish that thiamine influences tryptophan metabolism at stage *A* ... *A*, but they do not show whether or not the effect is direct, i.e. whether thiamine is functioning as part of an enzyme cofactor. The reaction involved is quite different in type from the reactions normally considered to be dependent on thiamine. But it is of some interest in this connexion that the two vitamin antagonists oxythiamine and neopyrithiamine have very different actions. Oxythiamine inhibits pyruvate decarboxylation without producing marked neurological changes, whereas neopyrithiamine produces the neurological symptoms without necessarily affecting pyruvate decarboxylation (Woolley & Merrifield, 1954). This strongly suggests participation of thiamine in reactions other than α-keto acid decarboxylation, normally considered to be its prime function.

The inhibition in thiamine deficiency of the first stage of tryptophan metabolism by the kynurenine–nicotinic acid pathway may assist in the study of other pathways by forcing metabolism to occur by alternative routes. There is of course no evidence that thiamine deficiency does not also affect these other pathways, and a complication may also arise if an increased blood α-keto acid level causes an abnormal degree of indolepyruvic acid formation by transamination.

The results obtained in biotin deficiency strongly suggest that in the rat biotin does not influence any stage in the conversion of tryptophan into hydroxykynurenine. The results thus disagree with the conclusions of Shanmuga Sundaram *et al.* (1954) on the function of biotin in tryptophan metabolism in *Neurospora*, in which they consider biotin to be a cofactor in formylkynurenine formation. There remains, however, the possibility, considered unlikely, that biotin is part of a coenzyme which is not readily dissociated from its enzyme in biotin deficiency in the rat. The Indian workers based their results on experiments with a vitamin antagonist, which implies that any such coenzyme is dissociable in *Neurospora*.

## SUMMARY

1. The metabolism of tryptophan has been studied in rats deficient in (*a*) thiamine, (*b*) thiamine and pyridoxine, (*c*) biotin, and (*d*) biotin and pyridoxine.

2. On imposing a thiamine deficiency on pyridoxine-deficient rats, and feeding supplementary tryptophan, the metabolite-excretory pattern typical of pyridoxine deficiency becomes abolished or much reduced. Formation of the substrates of the kynureninase reaction is therefore inhibited. Kynurenine fed at the same stage results in a marked metabolite excretion. No formylkynure-

nine is excreted on giving tryptophan to rats with a simple thiamine deficiency.

3. It is therefore concluded that thiamine is concerned in the conversion of tryptophan into formylkynurenine.

4. Similar experiments combining biotin and pyridoxine deficiencies suggest that biotin is unlikely to be concerned in any stage in the conversion of tryptophan into hydroxykynurenine.

5. Considerable advantages were found in giving vitamins by stomach-tube rather than by mixing them in the diet.

I thank Dr A. M. Copping for her advice, and Mr A. Asatoor and Miss R. Paul for skilled assistance.

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# Excretion of Conjugated 2-Amino-3-Hydroxyacetophenone by Man, and its Significance in Tryptophan Metabolism

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In a survey of aromatic metabolites in the urine of patients with various leukaemias and anaemias it was noticed that several of these urines contained appreciable amounts of a substance with a strong pale-blue fluorescence. This paper describes its identification.

## METHODS

Aromatic metabolites were isolated from the urines by adsorption on deactivated charcoal and subsequent phenol elution, as described in detail elsewhere (Dalgliesh, 1955*a*). Preparative chromatograms were run by the descending technique on Whatman 3 MM paper and other chromatograms similarly on Whatman no. 1 paper. Paper electrophoresis was carried out for 45–60 min. on Whatman no. 1 paper soaked in 0·4% (v/v) aqueous acetic acid with an applied potential gradient of 50 v/cm. Ultraviolet absorption spectra of eluates were determined with a Unicam spectrophotometer model SP. 500. The reagents used for determining reactive groups have been described elsewhere (Dalgliesh, 1955*a*).

The following solvents were used for chromatography: the organic phase of a *n*-butanol–glacial acetic acid–water mixture (4:1:5 by vol.); 'Phenol liquefactum' B.P.–water, 100:15 (v/v) ('neutral phenol'); *n*-pentanol–pyridine–water (7:7:6 by vol.); aqueous KCl (20%, w/v); aqueous sodium formate (5%, w/v).

*Persulphate oxidation of o-aminoacetophenone* (cf. Boyland & Sims, 1954). To *o*-aminoacetophenone hydrochloride (70 mg.) in 1·5 ml. water was added 0·9 ml. N-NaOH (approx. 2·2 equiv.) and sufficient acetone to give a clear solution. A solution of potassium persulphate (110 mg., 1 equiv.) in 10 ml. water was added, and the mixture set aside overnight. The brown reaction product was then diluted, filtered and acidified with acetic acid, and the products were adsorbed on deactivated charcoal. This was then eluted with phenol and the products were purified and examined by the procedures used for urinary metabolites.

## RESULTS

The unknown substance had a fluorescence very similar in colour to that of kynurenine. It moved on butanol–acetic acid chromatograms at a medium $R_F$ slightly faster than, but usually overlapping, indican, and in its turn slightly slower than, but overlapping, a non-fluorescent substance, positive to Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in dilute HCl), which will be the subject of a future communication. Preparative chromatography successively in butanol–acetic acid, neutral phenol and again in butanol–acetic acid, the appropriate areas being cut out and eluted at each stage, gave an extract in which no other substances could be detected chromatographically. The ultraviolet spectrum of the final eluate showed maxima at about 260 and 360 m$\mu$., characteristic of a

derivative of *o*-aminoacetophenone. The substance gave an orange colour with Ehrlich's reagent, as is to be expected for an *o*-aminoacetophenone with an unsubstituted amino group, but it did not give the ninhydrin reaction and could be separated from kynurenine on chromatography. On attempting to diazotize and couple (Ekman's reaction) a yellow colour appeared on diazotization (spraying with dilute HCl and nitrite). This behaviour recalled conjugates of hydroxykynurenine (Dalgliesh, 1952) and strongly suggested a 2-amino-3-hydroxyacetophenone skeleton.

The substance was stable in neutral solution, or in dilute acetic acid (it was not decomposed over several weeks in urines preserved with acetic acid, and was not hydrolysed on butanol–acetic acid chromatograms). It was readily hydrolysed by mineral acid. When a solution was made normal with respect to HCl and allowed to evaporate overnight in a vacuum desiccator over soda, conversion into a new material occurred. This ran fast on butanol–acetic acid chromatograms, had the typical greenish fluorescence of 3-hydroxykynurenine, but was separable from $N^{\alpha}$-acetyl-3-hydroxykynurenine. It was a strong reducing agent, reducing ammoniacal silver nitrate immediately, as is to be expected for a substance containing an unsubstituted *o*-aminophenol group. It also gave the expected orange-pink colour with Ehrlich's reagent.

It therefore seemed very probable that the metabolite was 2-amino-3-hydroxyacetophenone or a related substance conjugated on the phenolic group as the sulphate, phosphate or glucuronide. On paper electrophoresis in 0·4% acetic acid, conditions under which carboxyl dissociation is largely inhibited, the unhydrolysed material moved as a strong acid (indican and indoleacetic acid used for comparison; indican moved as a strong acid and indoleacetic acid remained close to the origin under the same conditions). This suggested a phenolic sulphate or phosphate, rather than a glucuronide. The hydrolysed material moved as a weak base. An HCl hydrolysate gave a marked precipitate with barium chloride solution. The conjugating group was therefore probably sulphate.

General considerations (see under Discussion) made it likely that the hydrolysed material was 2-amino-3-hydroxyacetophenone itself. An authentic sample of this substance was very kindly given by Professor L. Musajo (cf. Musajo, Spada & Casini, 1950) and hydrolysate and authentic substance were compared. The two behaved identically on paper chromatography in a variety of solvents, as shown in Table 1. The fluorescent material originally present in the urines was therefore considered to be 2-amino-3-hydroxyacetophenone *O*-sulphate, and this was confirmed by partial synthesis.

Table 1. *$R_F$ values of the urinary metabolite and related substances*

| | Developing solvent* | | | | |
|---|---|---|---|---|---|
| | Butanol–acetic acid | Neutral phenol | Pentanol–pyridine–water | 20% KCl | 5% Na formate |
| Urinary metabolite | 0·42† | 0·36 | 0·58 | 0·72 | 0·80 |
| Product of persulphate oxidation of *o*-aminoacetophenone | 0·42 | 0·36 | 0·58 | 0·72 | 0·79 |
| Hydrolysis product of urinary metabolite | 0·85 | 0·88 | 0·94 | 0·47 | 0·62 |
| Authentic 2-amino-3-hydroxyacetophenone | 0·85 | 0·88 | 0·95 | 0·48 | 0·61 |

* All on Whatman no. 1 paper by the descending technique. For composition of solvents see under Methods.

† $R_F$ values for some other substances in this solvent under comparable conditions are: kynurenine, 0·40; tryptophan, 0·44; indican, 0·34. In mixtures these values may be modified by mutual displacement.

Aromatic amines react with alkali persulphates to give *o*-aminophenylsulphates (Boyland & Sims, 1954). Treatment of *o*-aminoacetophenone with potassium persulphate readily gave a product giving the same colour reactions as the urinary metabolite and travelling identically in several chromatographic solvents (Table 1) as well as on paper electrophoresis. Besides this major product there was also a minor product, very similar in chemical behaviour but differing in $R_F$ values, together with free aminohydroxyacetophenones. In an investigation of the reaction of persulphates with anthranilic acid (S. F. Contractor & C. E. Dalgliesh, unpublished), which was made to investigate possible application of this reaction to large-scale synthesis of hydroxykynurenine and related compounds, it was found that besides the major product, 3-hydroxyanthranilic acid sulphate (considered by Boyland & Sims to be the only phenolic sulphate produced), the isomeric 5-hydroxyanthranilic acid sulphate was also formed, together with free 3- and 5-hydroxyanthranilic acids. The minor product in the reaction of persulphate with *o*-aminoacetophenone may therefore

well be 2-amino-5-hydroxyacetophenone *O*-sulphate, but it has not been further investigated. As only a small amount of *o*-aminoacetophenone was available, the reaction could not be carried out on a sufficiently large scale to make practicable isolation of the products in the crystalline state.

## DISCUSSION

The only known likely source of a derivative of 2-amino-3-hydroxyacetophenone is tryptophan, which is metabolized via 3-hydroxykynurenine (I, Fig. 1; see Dalgliesh, 1955*b*, for collected references). Possible changes in the side chain of this substance are shown in Fig. 1. Aminohydroxyacetophenone (VI) might arise by decarboxylation being formed. This reaction requires pyridoxal phosphate as coenzyme, as does the transamination of hydroxykynurenine leading to xanthurenic acid. It seems very probable that these reactions involve formation of a Schiff's base which can tautomerize, as follows (where Ar represents the 2-amino-3-hydroxyphenyl group and Py.CHO represents pyridoxal phosphate: cf. Metzler, Ikawa & Snell, 1954; Longenecker & Snell, 1955; Dalgliesh, 1955*b*):

$$\mathrm{Ar.CO.CH_2.CH.CO_2H} \rightleftharpoons \mathrm{Ar.CO.CH_2.C.CO_2H}$$
$$\qquad\qquad\quad \mathrm{N{=}CH.Py} \qquad\qquad\qquad\quad \mathrm{N{-}CH_2.Py}$$

The latter form of the Schiff's base (which *in vivo* may be coordinated with a metal and protein) is

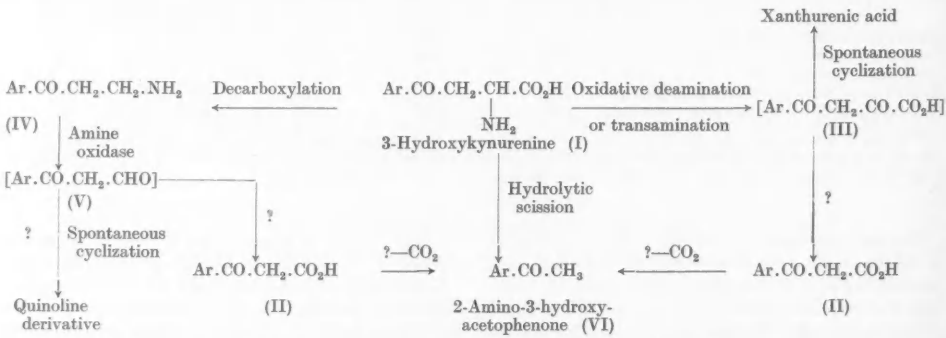


Fig. 1. Possible routes for formation of 2-amino-3-hydroxyacetophenone from 3-hydroxykynurenine. Ar represents the 2-amino-3-hydroxyphenyl residue.

of the corresponding substituted acetic acid (II). But neither of the routes for formation of the latter substance generally applicable in amino acid metabolism is likely in the present case. Formation of the acetic acid derivative from the corresponding pyruvic acid (III) is unlikely, as the pyruvic acid is known to cyclize spontaneously to xanthurenic acid (4:8-dihydroxyquinoline-2-carboxylic acid) (Musajo, Spada & Bulgarelli, 1950). Similarly, formation via the corresponding amine (IV) and aldehyde (V) is unlikely, as the aldehyde would also be expected to undergo spontaneous cyclization. Thus the related kynurenamine is almost quantitatively excreted as quinoline derivatives by the rat (Butenandt & Renner, 1953). The only other likely route for formation of the acetophenone is by a direct hydrolytic scission of the hydroxykynurenine side chain. Direct scission on the other side of the methylene carbon is the normal route for hydroxykynurenine metabolism by the enzyme kynureninase, hydroxyanthranilic acid and alanine analogous in electron distribution to $\beta$-keto acids and $\beta$-diketones. Direct scission of the hydroxykynurenine side chain to give the acetophenone derivative is thus analogous to the formation of acetone from acetoacetic acid. Excretion of the acetophenone derivative may therefore be due to a small spontaneous decomposition of the hydroxykynurenine–pyridoxal phosphate complex, or the reaction may be enzymically accelerated. The fact that the acetophenone derivative is excreted as the phenolic sulphate, a typical 'detoxication' product, suggests that the reaction is unlikely to be one that is metabolically useful.

Hydroxyaminoacetophenone was first observed in pathological urines. It is also excreted by at least some normal persons, though in amounts so small as to be readily overlooked. Too many at present unknown factors exist in the isolation procedure to allow determination of excretion values, but it appears most unlikely that normal excretion by man is as much as 1 mg./24 hr. The

relationship between the increased excretion and the fundamental lesion in the pathological cases, and the range of pathological cases in which this increased excretion occurs, remain to be determined.

## SUMMARY

1. A metabolite excreted in small amount in some normal human urines, and in appreciably larger amounts in certain pathological urines, is shown by degradative and synthetic evidence to be 2-amino-3-hydroxyacetophenone *O*-sulphate.

2. Reasons are given for considering this substance to arise by hydrolytic scission of the side chain of hydroxykynurenine.

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# Studies on the Acetylesterase of *Sclerotinia laxa*

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This paper deals with the hydrolysis of phenolic esters by enzyme preparations from *Sclerotinia laxa* Aderh. & Ruhl., one of the fungi causing the brown-rot diseases of fruits. The investigations were undertaken on account of the apparent role of a fungal enzyme in the mode of fungistatic action of esters of 2:3-dichloro-1:4-naphthaquinone (Byrde & Woodcock, 1952, 1953).

Macdonnell, Jang, Jansen & Lineweaver (1950), in the course of studies of pectin and acetylesterases of fruits of *Citrus* spp., showed also that a pectin esterase of fungal origin readily hydrolysed phenyl acetate and phenyl propionate at pH 6·8, and, to a lesser extent, at pH 4·5. Subsequently Jermyn (1953) demonstrated that three or four components with esterase activity could be separated by paper chromatography from cultures of *Aspergillus flavus-oryzae*. In further studies on the enzymes of this fungus, Crewther & Lennox (1953) showed that a pH of 6·0 was the optimum for esterase activity, and demonstrated that there was little or no activity in cultures under 6 days old.

Amongst the more numerous workers on esterases of non-fungal origin, Aldridge (1953), in a study of serum esterases, classified them into two types, on the basis of substrate specificity and their inhibition by diethyl *p*-nitrophenyl phosphate (paraoxon). He defined type A esterases as those hydrolysing *p*-nitrophenyl acetate more readily than the corresponding butyryl ester, while relatively resistant to inhibition by paraoxon. Type B esterases, on the other hand, hydrolysed the butyryl derivative more rapidly than the acetate, and were inhibited by concentrations of paraoxon as low as $10^{-7}$–$10^{-8}$ M.

The effect of nuclear substitution on the hydrolysis of phenyl acetate by a wheat-germ esterase was studied by Gawron, Grelecki & Duggan (1953), who suggested that electromeric factors might account for the differing rates of hydrolysis. Electron-withdrawing substituents, especially in the *meta* position, increased the hydrolysis rate, but electron-donating substituents decreased the rate.

## EXPERIMENTAL

### *Source of enzyme*

The enzyme used in most of the experiments was a crude preparation from cultures of *S. laxa* grown on autoclaved acidified potato plugs, the medium used in this laboratory for routine production of spores. The mycelium was scraped from the surface of the cultures and macerated with buffer solution or water, according to the method subsequently used for the estimation of enzyme activity. The resultant pulp was filtered under suction and the crude filtrate stored at −4°. The enzyme was roughly concentrated by successive freezing and thawing and removal of ice crystals, resulting in a preparation of moderately high activity.

Preparations of higher activity could be obtained by fractionation with ammonium sulphate in the presence of sodium oxalate, followed by dialysis, as described by Jansen, Jang & Macdonnell (1947).

### *Substrates*

The following esters were prepared by conventional methods: phenyl acetate, b.p. 196°; *o*-chlorophenyl acetate, b.p. 103°/15 mm.; *p*-chlorophenyl acetate, b.p. 76°/0·8 mm.; 2:4:5-trichlorophenyl acetate, m.p. 66–67·5°; 2:4:6-trichlorophenyl acetate, m.p. 47–48°; pentachlorophenyl acetate, m.p. 147–149°; *p*-chlorophenyl propionate, b.p. 160–164°/8 cm.; *p*-chlorophenyl butyrate, b.p. 172–176°/8 cm.; *p*-chlorophenyl valerate, b.p. 190·8°/9 cm.; *p*-chlorophenyl hexanoate, b.p. 108–112°/0·4 mm.; *p*-chlorophenyl benzoate, m.p. 87–88°.

In order to disperse the esters in water without using a mutual solvent, each was formulated as a 20% wettable preparation on a kaolin base, by slurrying an acetone solution with kaolin and grinding the suspension in a mortar until the solvent had evaporated.

### *Estimation of enzyme activity*

(*a*) *Colorimetric method.* This technique was similar to that described by Jansen, Nutting & Balls (1948). It was based on the colorimetric estimation of *o*-nitrophenol liberated by the enzymic hydrolysis of *o*-nitrophenyl acetate.

The test system comprised 9 ml. suitable buffer solution, 1 ml. enzyme preparation (in buffer), and 0·2 ml. 0·1M *o*-nitrophenyl acetate (in ethanol). The development of yellow colour was estimated on a Spekker absorptiometer, a violet filter (Ilford 601, max. transmission approx. 4300 Å) being used, and with a water setting of 1·300. Necessary blanks were included. The amounts of *o*-nitrophenol liberated were estimated from a standard curve.

*p*-Nitrophenyl derivatives have also been used by other workers (Huggins & Lapides, 1947; Jermyn, 1953; Crewther & Lennox, 1953), but all such colorimetric methods are restricted to use with esters of coloured phenols and at pH values below 8·5, owing to excessive non-enzymic hydrolysis of the esters under alkaline conditions.

A modification of this method used for detection of esterase activity *in vivo* and in chromatographic studies depended on the hydrolysis of diacetylfluorescein to fluorescein, which, unlike its ester, fluoresces strongly in ultraviolet light.

(*b*) *Manometric method.* This technique has also found wide use in esterase studies (Gawron *et al.* 1953; Aldridge, 1953). It is based on the manometric estimation of $CO_2$ liberated from $NaHCO_3$ by the carboxylic acid formed on enzymic hydrolysis of the ester.

Warburg flasks (15 ml.) with single side arm were used. The main flask contained 0·75 ml. 0·03M-$NaHCO_3$ and 1·50 ml. substrate (1% aqueous suspension of a 20% w/w preparation on kaolin). The side arm contained 0·95 ml. enzyme preparation made up in water and with pH adjusted to 7·0.

The flasks were gassed with 5% $CO_2$ in $N_2$ and equilibrated at 25° before addition of the contents of the side arm. The molarity of the $NaHCO_3$ was such as to give a pH of 7·0 in equilibrium with an atmosphere of 5% $CO_2$ (Umbreit, Burris & Stauffer, 1949, p. 26). Necessary blanks were included.

This method, while not restricted to esters of any specific phenol, is limited to the pH range 6·4–7·8 by virtue of the $NaHCO_3$ buffer. Gawron *et al.* (1953) also pointed out that it is necessary to use a correction factor for carbon dioxide liberated as a result of dissociation of the free phenol. This factor is expressed by $(K_a+[H^+])/(2K_a+[H^+])$, where $K_a$ is the dissociation constant of the free phenol. At a pH of 7·0 this factor is negligible for the less acidic phenols but becomes appreciable for trichlorophenols and pentachlorophenol.

### *Chromatographic separations*

The separation of the esterase of *S. laxa* was carried out by the method of Jermyn (1953), descending paper chromatography being used. The solvent selected was 40% acetone buffered to pH 4·2 with McIlvaine citrate–phosphate buffer (0·01–0·02M). Whatman no. 4 paper was used to ensure rapid development. The papers were removed from the trough and dried at laboratory temperature when the solvent front had moved to a distance of 20 cm. from the initial spot. As soon as they were dry, they were sprayed with diacetylfluorescein (0·01% in 50%, v/v, aqueous acetone buffered to pH 6·5), and after 30 min. exposed to ultraviolet light to make the spots visible.

## RESULTS

### *Substrate specificity*

*Effect of side chain.* The relative activity of the enzyme preparation from *S. laxa* towards different side chains on the phenol nucleus was investigated by the manometric technique, with a series of *p*-chlorophenyl esters as substrates. Table 1 summarizes the results obtained in duplicate tests and shows that increasing side-chain length is associated with decreasing enzyme activity towards the ester.

Table 1. *Relative esterase activity of an extract from* Sclerotinia laxa *towards esters of* p*-chlorophenol*

Hydrolysis of the esters in the absence of the enzyme preparation was of the order of 2% of that in its presence. Mean rate of enzymic hydrolysis of acetyl ester: 11·45 $\mu$moles/ml. enzyme prep./hr. For conditions see text.

| Ester | Relative activity |
|---|---|
| Acetyl | 100 |
| Propionyl | 66 |
| Butyryl | 28 |
| Valeryl | 10 |
| Hexanoyl | 2 |
| Benzoyl | 2 |

*Effect of nucleus.* The relative activity of the esterase towards a range of chlorinated phenyl acetates of interest as fungistatic agents was investigated by the manometric technique, with phenyl acetate as a standard in each test. Table 2 summarizes the results obtained in duplicate experiments; the $K_a$ values used for corrections for phenolic dissociation are also listed.

Table 2. *Relative esterase activity of an extract from* Sclerotinia laxa *towards acetyl esters of chlorinated phenols*

Hydrolysis of the esters in the absence of enzyme was of the order of 3% of that in its presence. Mean rate of enzymic hydrolysis of phenyl acetate: 9·57 μmoles/ml. enzyme prep./hr. For conditions see text.

| Parent phenol | $10^7 K_a$* | Relative esterase activity (corr.) |
|---|---|---|
| Phenol | 0·001 | 100 |
| *o*-Chlorophenol | 0·36 | 93 |
| *p*-Chlorophenol | 0·21 | 74 |
| 2:4:5-Trichlorophenol | 0·47 | 47 |
| 2:4:6-Trichlorophenol | 3·80 | 12 |
| Pentachlorophenol | 55·00 | <1 |

* After Tiessens (1931).

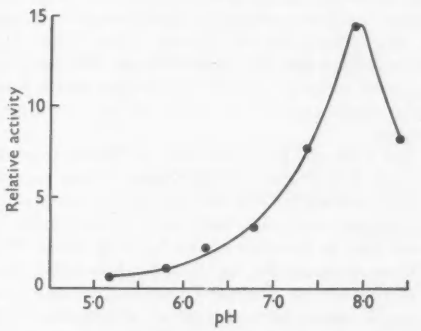


Fig. 1. Effect of pH on the activity of *S. laxa* acetylesterase. For conditions see text.

### pH *relationships*

The effect of pH on enzyme activity was studied by the colorimetric method, with a McIlvaine citrate–phosphate buffer. Fig. 1 illustrates the relative activity of the enzyme over the pH range 5·2–8·4; no measurable activity was found below pH 5·0. The optimum occurs in the region of pH 7·8, a value higher than that obtained for the preparation from *A. flavus-oryzae* by Crewther & Lennox (1953).

### *Inhibitory effect of diethyl* p*-nitrophenyl phosphate*

The effect of diethyl *p*-nitrophenyl phosphate (paraoxon) on the esterase from *S. laxa* was investigated by the colorimetric method, with a 0·1 M phosphate buffer (pH 6·65). The inhibitor (0·1 ml. of an acetone solution of appropriate concentration) was added to the enzyme (1 ml.) and buffer (9 ml.) 30 min. before the addition of substrate (0·2 ml.). The results of percentage inhibition of enzyme activity, measured over a 30 min. period, were transformed into probit units (Finney, 1952) and plotted against the logarithm of final inhibitor concentration. Fig. 2 illustrates the results obtained, together with those of similar experiments with enzymes of plant and insect origin. Clearly, the fungus enzyme, by contrast with the insect enzyme, is relatively tolerant of paraoxon.

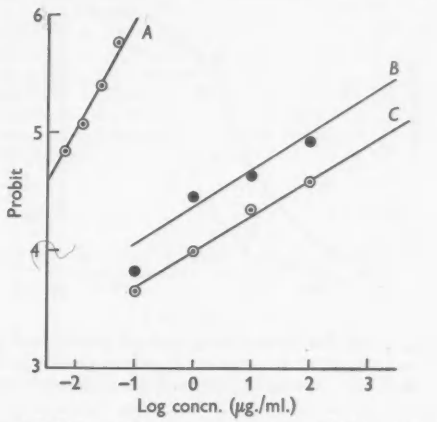


Fig. 2. Inhibtion of acetylesterases by diethyl *p*-nitrophenyl phosphate. For conditions see text. *A, Locusta-migratoria migratorioides* ($8 \times 10^{-3}$); *B, Vicia faba* (100). *C, Sclerotinia laxa* ($2{\cdot}2 \times 10^3$). Approximate dosages for 50% inhibition (μg./ml.) are shown in brackets.

### *Exocellular enzyme production by developing cultures*

Cultures of *S. laxa*, from a heavy spore inoculum, were grown on 50 ml. portions of a liquid potato–dextrose medium (200 g. boiled potato, 20 g. dextrose, 1 l. water) in 250 ml. conical flasks. At intervals of a few days after inoculation the dry weight of duplicate mycelial mats was determined. At the same time, the esterase activity of the culture filtrates was estimated by the colorimetric method, with a 0·1 M phosphate buffer (pH 6·65).

Fig. 3 shows the results obtained, which indicate that exocellular esterase secretion lagged behind mycelial growth; no measurable enzyme activity developed before the sixth day. This is in accordance with the findings of Crewther & Lennox (1953), using *A. flavus-oryzae*.

In a further test, acetylesterase activity was assessed *in vivo* under the experimental conditions commonly used for toxicity trials in this laboratory. Four plates were poured from 50 ml. melted dextrose–peptone–phosphate agar (pH 6·5) to which had been added 0·5 ml. of a solution of

diacetylfluorescein (1% in acetone). On cooling, they were inoculated at a central point with a disk (4 mm. diameter) from a culture of *S. laxa* and incubated at 25°. After 1 day a strongly fluorescent ring had developed round the disk, from which the mycelium subsequently grew out, surrounded by a fluorescent zone about 1 cm. wide. The background showed only slight fluorescence. This result

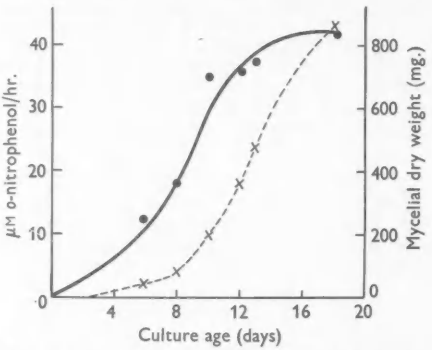


Fig. 3. Relation between acetylesterase activity and age and weight of mycelium of *S. laxa* in liquid culture. ●, Weight; ×, enzyme activity. For conditions see text.

suggests that, under these experimental conditions, hydrolysis of diacetylfluorescein by an exocellular esterase occurred at an early stage in the growth of the fungus, and in the proximity of the mycelium was considerably in excess of non-enzymic hydrolysis.

*Chromatographic separation of enzyme components*

A crude *S. laxa* extract was separated into four components showing acetylesterase activity. The distances (cm.) of these spots from the starting line, after 20 cm. solvent travel, were as follows: (1) ±0·5, (2) 6·0–7·5, (3) 12·0–13·5, (4) 16·5–20·0.

Chromatography of fractions obtained by ammonium sulphate precipitation showed that, whilst the enzyme had been concentrated, the fractionation had only partially separated these four components.

An attempt was also made to examine chromatographically an esterase preparation from the gut of *Locusta*. Excessive streaking made evaluation difficult, but little evidence of difference was obtained, other than the absence of component (2).

## DISCUSSION

The results suggest that the esterase of *Sclerotinia laxa* resembles the type A serum esterase described by Aldridge (1953). The fungal enzyme hydrolysed *p*-chlorophenyl acetate more readily than the butyryl derivative, and was relatively little affected by diethyl *p*-nitrophenyl phosphate.

The demonstration in cultures of *S. laxa* of an active esterase, with a range of substrates, lends support to the suggestion put forward by Byrde & Woodcock (1953, 1955) that the activity of esters of both 2:3-dichloro-1:4-naphthahydroquinone and pentachlorophenol against several fungi (including *S. laxa*) might arise from their enzymic hydrolysis to the toxic phenol. Moreover, in both series, the lower fatty acid esters showed greater fungistatic activity than the higher members of the series, whilst it has now been demonstrated manometrically, for a series of *p*-chlorophenyl esters, that increasing length of aliphatic side chain is associated with a decreasing rate of enzymic hydrolysis. The statistically significant effect of pH, over the range 5·4–7·5, on the fungistatic activity of two pentachlorophenyl esters towards *S. laxa* (Byrde & Woodcock, 1956) may similarly be ascribed to differences in the rates of enzymic hydrolysis over this range, which have been demonstrated *in vitro* by a colorimetric method.

The evidence presented also indicates that the primary site of action of the fungal enzyme in such toxicity tests is outside the cell, rather than within, and that non-enzymic hydrolysis plays a relatively small part in bringing about the fungistatic effect of these compounds. On the other hand, the possibility cannot be dismissed that hydrolysis of the phenolic esters may also occur within the fungal cell after penetration by molecules of the esters. Such a mode of action was suggested by Grove (1953) to account for the fungistatic activity of acetylgladiolic acid against conidia of *Botrytis allii* Munn.

## SUMMARY

1. The presence of an acetylesterase of *Sclerotinia laxa* was demonstrated. The activity of the enzyme towards phenolic esters of fatty acids decreased with increasing molecular weight of the side chain, and was also related to the nature of the phenolic nucleus.

2. The esterase showed optimum activity at pH 7·8, and was relatively resistant to inhibition by diethyl *p*-nitrophenyl phosphate. Exocellular enzyme activity of developing cultures of *S. laxa* in a liquid medium lagged behind mycelial growth, but localized production was demonstrated at an early stage in cultures on solid medium.

3. On the basis of these results, the enzyme appears akin to type A serum esterases.

4. From the crude extract of *S. laxa* four components with esterase activity were separated by paper chromatography.

5. Exocellular fungal esterase appears to play a large part in bringing about the fungistatic activity of phenolic esters.

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# Changes in Lens During the Formation of X-Ray Cataract in Rabbits

## 3. PHOSPHATES*

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The first biochemical change caused by irradiation of tissues is unknown. There is some evidence, reviewed by Ord & Stocken (1953), that changes take place in the high-energy phosphate ($\sim$P) compounds but assessment of the results is complicated by the fact that in most situations it is difficult to separate primary from secondary effects. Gross histological change may occur in an organ within a few hours of whole-body irradiation, with lysis of some cells and influx of others from the blood stream. No valid biochemical comparison is possible between the tissue in this state and in the normal state before irradiation. Where cell population has changed one can expect large changes in chemical and enzymic make-up.

From this point of view a study of the effect of irradiation on the lens may be peculiarly valuable. It is an isolated non-vascular organ, derived from a single type of embryonic ectodermal cell, and any observed early effect of radiation is more likely to be a primary effect than it is in other tissues where the cell population is complex to start with and may change after irradiation. We have already shown that certain changes in the composition and enzymes of lens occur during the early stages of the formation of X-ray cataract (Pirie, van Heyningen & Boag, 1953; van Heyningen, Pirie & Boag, 1954). The changes may not be primary effects, however, since their onset occurred some weeks after irradiation. Since changes in $\sim$P may be an early result of irradiation, we have examined the effect of X-rays on the phosphate compounds of the lens.

We have used rabbits of 14–28 days in this study for two reasons. First, it is known that the concentration of high-energy phosphate compounds is greater in the lens of the young animal than it is in the old (Nordmann & Mandel, 1952; Müller & Kleifeld, 1953) and, secondly, the development of cataract for a given dose of X-rays is far quicker in the young (Cogan & Donaldson, 1951). Both these characteristics are probably related to the higher proportion of dividing cells and nucleated fibres in the young lens. The irradiated lens was compared directly with its normal fellow, as it has been shown that the composition and weight of the two lenses of a single rabbit are very similar, although values may differ widely from animal to animal.

A known early change in the rabbit lens after X-ray treatment is a fall in glutathione (Pirie *et al.* 1953; van Heyningen *et al.* 1954). We have therefore estimated glutathione in some of the lenses, considering that any change in it was a yard-stick by which to assess other changes.

* Part 2: van Heyningen, Pirie & Boag (1954).

## METHODS

*Irradiation.* Dutch rabbits were used. They were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (Nembutal) and placed in a sitting position. The X-ray beam of diameter 1·4 cm. was directed vertically from above on the right eye of the rabbit, which was placed so that the judged position of the lens was in the centre of the path of the beam. With this position of the rabbit and this diameter and direction of the beam, little or no radiation reached the left lens. The rabbits were used when 13–17 days old, the right eye of each of the young in a litter being irradiated with a dose of 1000 r. Otherwise the technique used was the same as before (Pirie *et al.* 1953).

*Preparation of lens extracts:* (1) *Liquid air.* The rabbit was deeply anaesthetized with Nembutal, and then dropped head first into liquid air. The eyes were chiselled out of the head and the lens dissected out from each eye, dropped into a tared tube containing 1·0 ml. ice-cold 5% (w/v) trichloroacetic acid (TCA) and ground up. The different parts of the eye separated as the temperature rose slightly, and it was possible to get the lens into the acid before it melted. (2) *Ice-cold.* The rabbit was killed by Nembutal injection and the eyes were immediately removed and placed in ice-cold water for a few minutes. Each lens was then dissected out as quickly as possible and ground up in 1·0 ml. ice-cold 5% TCA.

The lens was weighed by difference in the TCA after it had been ground. The suspension was then centrifuged at 7000 *g* for 5 min. The supernatant fluid was removed, the largest possible sample was added to 0·3 ml. aminotrishydroxymethylmethane buffer at pH 7·4, 0·1 M, and the extract neutralized with 2% (w/v) KOH from a burette. The extract was kept chilled throughout, and after neutralization was stored at −5°. All determinations were completed within a few hours of extraction of the lens.

*Phosphorylated sugars and the energy-rich phosphate groups (~P) of adenosine triphosphate (ATP) and adenosine diphosphate (ADP).* These were determined by the enzymic method of Slater (1953). As little as 0·02 μmole ~P can be estimated with considerable accuracy by this method. It is therefore very suitable when, as in this case, only a small amount of material is available. The method depends on the enzymic formation of dihydroxyacetone phosphate, which oxidizes reduced diphosphopyridine nucleotide (DPNH) in the presence of glycerophosphate dehydrogenase. The amount of DPNH oxidized was measured spectrophotometrically, the reaction being almost complete in 3–5 min. The following reactions occur:

(1) Glucose 1-phosphate → glucose 6-phosphate → fructose 6-phosphate (phosphoglucomutase and hexose monophosphate isomerase).

(2) Fructose 6-phosphate + ATP → hexose diphosphate (HDP) + ADP (phosphohexokinase).

(3) 2ADP → ATP + adenylic acid (myokinase).

(4) HDP → glyceraldehyde phosphate + dihydroxyacetone phosphate (aldolase).

(5) Glyceraldehyde phosphate → dihydroxyacetone phosphate (triose phosphate isomerase).

(6) Dihydroxyacetone phosphate + DPNH → glycerophosphate + $DPN^+$ (glycerophosphate dehydrogenase).

The overall reaction is as follows:

$$HMP + {\sim}P + 2DPNH \rightarrow 2 \text{ glycerophosphate} + 2DPN^+,$$

where HMP is the sum of the three hexose monophosphates, and ~P represents the high-energy phosphate groups of ATP and ADP (and creatine phosphate; see below).

All the enzymes necessary for the reactions are contained in two preparations from rabbit muscle (Racker, 1947; Slater, 1953).

The method can be used in two ways: (*a*) in the presence of excess of ~P the disappearance of DPNH is a measure of the total concentration of HMP, HDP, pyruvic acid, phosphopyruvic acid and phosphoglyceric acid (PGA). These two last are converted by the enzymes present into pyruvic acid, which oxidizes DPNH in the presence of lactic dehydrogenase; (*b*) in the presence of excess of HMP

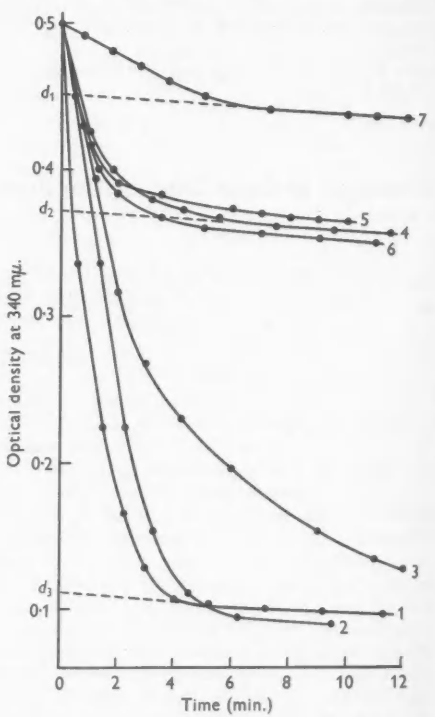


Fig. 1. Oxidation of DPNH by Slater's procedure (*b*). The complete reaction mixture contained DPNH (0·22 μmole approx.), glucose 6-phosphate (0·5 μmole), ATP (0·01 μmole), CP (0·03 μmole approx.), $MgCl_2$ (7·5 μmoles), aminotrishydroxymethylmethane buffer pH 7·6 (30 μmoles), suitable dilutions of creatine phosphokinase and Slater's two muscle enzyme extracts; final volume 3·0 ml. Curve 1, complete mixture; curve 2, complete mixture + $10^{-5}$ M iodosobenzoate; curve 3, complete mixture + $10^{-4}$ M iodosobenzoate; curve 4, complete mixture + $10^{-3}$ M iodosobenzoate; curve 5, complete mixture + $10^{-2}$ M iodosobenzoate; curve 6, CP omitted; curve 7, ATP and CP omitted (i.e. blank). $d_2 - d_1$ gives decrease in optical density due to ATP, $d_3 - d_1$ that due to ATP + CP and $d_3 - d_2$ that due to CP.

the disappearance of DPNH is a measure of ~P contained in ATP and ADP and of HDP, PGA, phosphopyruvic and pyruvic acids. We have shown by method (*a*) that only small amounts of these last four compounds are present in lens extracts (see Results) and hence the estimation gives a measure of the ~P of ATP and ADP in lens.

*Creatine phosphate* (*CP*). This was included in the substances determined in procedure (*b*) by the addition of creatine phosphokinase prepared from rabbit muscle (Kuby, Noda & Lardy, 1954). Insufficient creatine phosphokinase is present in the original enzyme preparations.

There was sufficient material from a single lens for most estimations to be done in duplicate. The lens weights ranged from about 90 mg. at 14 days to about 140 mg. at 29 days old. The results are calculated in terms of 100 g. wet weight of lens.

## RESULTS

### *Estimation of ~P of ATP, ADP, and CP*

Total high-energy phosphate was determined by procedure (*b*) and the results are given in Table 1.

Table 1. *High-energy phosphate in normal and irradiated rabbit lens*

The right eye of the irradiated rabbits received 1000 r.

| Normal rabbits | Days from irradiation | Age at death (days) | ~P (μm-moles/lens) R | ~P (μm-moles/lens) L | Difference (%) $\left(\frac{R-L}{L}\times 100\right)$ |
|---|---|---|---|---|---|
| *A* | — | 13 | 626 | 614 | +2·0 |
| *B* | — | 14 | 642 | 616 | +4·2 |
| *C* | — | 14 | 614 | 642 | −4·4 |
| *D* | — | 15 | 693 | 705 | −1·7 |
| *E* | — | 17 | 662 | 677 | −2·2 |
| *F* | — | 21 | 618 | 675 | +7·5 |
| *G** | — | 28 | 608 | 596 | +2·0 |
| Irradiated rabbits | | | | | |
| 46 | 0·25 | 13 | 620 | 654 | −5·2 |
| 47 | 1 | 18 | 677 | 669 | +1·2 |
| 48* | 1 | 16 | 417 | 475 | −12·2 |
| 49 | 1 | 16 | 648 | 680 | −4·7 |
| 50 | 2 | 19 | 862 | 891 | −3·3 |
| 51* | 2 | 17 | 536 | 579 | −7·4 |
| 52 | 2 | 17 | 632 | 657 | −3·8 |
| 53 | 3 | 18 | 710 | 735 | −3·4 |
| 54 | 4 | 17 | 640 | 600 | +6·7 |
| 55* | 4 | 19 | 640 | 663 | −3·5 |
| 56 | 4 | 21 | 628 | 825 | −23·9 |
| 57 | 6 | 23 | 925 | 963 | −3·9 |
| 58 | 7 | 24 | 796 | 784 | +1·5 |
| 59* | 7 | 22 | 677 | 755 | −10·3 |
| 60* | 9 | 26 | 590 | 783 | −24·6 |

* Killed with liquid air.

Creatine phosphate, in the presence of the kinase, reacts with ADP to form ATP, and the enzymic analysis of ~P will therefore include creatine phosphate. It was found that potassium *o*-iodosobenzoate inhibited creatine phosphokinase completely when at a concentration of $10^{-3}$ M, without affecting the activity of the muscle enzymes; therefore by addition of iodosobenzoate to the enzyme preparations a value for ~P of ATP and ADP alone could be obtained. Thus to get a value for creatine phosphate alone two estimations are necessary, one of total ~P and one of ~P without CP. Fig. 1 shows the results of such an experiment.

*Creatine and creatine phosphate.* These were also determined chemically by the method of Ennor & Rosenberg (1952).

*Inorganic phosphate.* This was determined by the method of Fiske & Subbarow (1925).

*Glutathione.* This was determined in the TCA extracts before neutralization, by the method of Grunert & Phillips (1951).

The numbers of the irradiated rabbits are consecutive with those in the two previous papers in the series (Pirie *et al.* 1953; van Heyningen *et al.* 1954). The table shows that, with four exceptions, the differences in high-energy phosphate between 15 pairs of irradiated and normal lenses were no greater than the differences found between pairs of normal lenses. A significant fall of ~P occurred in the irradiated lens of only one rabbit during the first 3 days after irradiation (rabbit 48; decrease 12·2%). From 4 to 9 days after irradiation decreases of 23·9, 10·3 and 24·6% were found in three rabbits; in the remaining four rabbits there was no significant change. One can say therefore that no systematic fall in ~P can be detected immediately after or within a few days of irradiation. We have found that there is a great fall in ~P in complete X-ray cataract (unpublished results); this confirms

the work of Nordmann & Mandel (1953) on diabetic cataract in rats, and it is probable that the falls observed at 4–9 days after irradiation are the beginning of this decline. Rabbits 21 days old irradiated with 1500 r show the first signs of cataract 15–20 days later (Cogan & Donaldson, 1951); therefore a nine-day period is a considerable part of the time needed for clinical cataract production.

Table 2. *Creatine phosphate in rabbit lens and brain, determined enzymically*

The right eye of the irradiated rabbits received 1000 r.

| | Days from irradiation to death | Age at death (days) | Creatine phosphate in lens (μm-moles/lens) R | L | Creatine phosphate in lens (μmoles/100 g.) R | L | Creatine phosphate in brain (μmoles/100 g.) |
|---|---|---|---|---|---|---|---|
| Normal rabbits | | | | | | | |
| *F* | — | 17 | 38 | 3 | 37 | 2 | — |
| *G** | — | 28 | 15 | 40 | 11 | 30 | 140 |
| *H* | — | *ca.* 440 | 108 | 98 | 18 | 18 | — |
| Irradiated rabbits | | | | | | | |
| 47 | 1 | 18 | 33 | 24 | 28 | 18 | — |
| 48* | 1 | 16 | 30 | 13 | 33 | 13 | — |
| 50 | 2 | 19 | 44 | 44 | 33 | 34 | — |
| 51* | 2 | 17 | 72 | 111 | 71 | 99 | — |
| 56 | 4 | 21 | 0 | 25 | 0 | 20 | — |
| 55* | 4 | 19 | 32 | 0 | 29 | 1 | — |
| 59* | 7 | 22 | 51 | 21 | 41 | 17 | — |
| 60* | 9 | 26 | 51 | 0 | 29 | 0 | 282 |

* Killed with liquid air.

Table 3. *Creatine and creatine phosphate in normal rabbit and bovine lens*

| Animal | Free creatine (μmoles/100 g.) Chemical determination | Creatine phosphate (μmoles/100 g.) Chemical determination | Creatine phosphate (μmoles/100 g.) Enzymic determination |
|---|---|---|---|
| Calf | 58 | 18 | — |
| Cow | 6 | 9 | — |
| Rabbit | | | |
| 12 days old* (4 lenses) | 34 | 5 | 9 |
| 15 weeks old (2 lenses) | 12 | 3 | — |

* Killed with liquid air.

*Estimation of creatine phosphate*

An attempt was made to determine creatine phosphate in lens by enzymic methods using *o*-iodosobenzoate as an inhibitor of creatine phosphokinase. Creatine phosphate could be satisfactorily determined in brain in this way, but the results of the determinations in lens were quite erratic, the apparent creatine phosphate being as great in a lens that had been extracted at room temperature as in one that had been frozen (Table 2). Chemical determination of free and combined creatine in bovine and rabbit lens (Table 3) showed that part is in a combined form, presumably creatine phosphate, but the amount is too small for determination enzymically in the presence of large amounts of other reactive phosphorus compounds.

*Determination of HMP, HDP, PGA, phosphopyruvic and pyruvic acids*

Procedure (*a*) was used to determine these substances in lens extracts. Table 4 shows that there is no consistent difference between irradiated and normal lens. The total amount of these substances in the lens is small, and the accuracy of the determination is therefore lower than that of ~P.

*Determination of inorganic phosphate*

The inorganic phosphate in normal lens is a small fraction of the total and we considered that it was worth while to determine whether any change took place in this fraction after irradiation. One complete lens had to be used for each estima-

tion, and Table 5 shows that it was impossible to detect any consistent change in inorganic phosphate after irradiation.

*Determination of glutathione (GSH)*

Table 6 sets out the values obtained in normal and irradiated lens. In these young rabbits, there does seem to be a very early fall in GSH. In older rabbits the fall in GSH is a later change but opacity formation is also a far more prolonged process. The rise in total GSH with lens growth, which is very rapid at this stage, can be readily seen from Table 6, and may be contrasted with the much more constant content of ~P (Table 1).

Table 4. *Hexose monophosphates and hexose diphosphate, phosphoglyceric, phosphopyruvic and pyruvic acids in normal and irradiated lenses of rabbits*

The right eye of the irradiated rabbit received 1000 r.

| Irradiated rabbits | Days from irradiation to death | Age at death (days) | HMP, HDP, etc. (μm-moles/lens) R | HMP, HDP, etc. (μm-moles/lens) L | Difference (%) $\left(\frac{R-L}{L}\times 100\right)$ |
|---|---|---|---|---|---|
| 46 | 0·25 | 13 | 41 | 43 | −4·7 |
| 54 | 4 | 17 | 56 | 56 | 0 |
| 57 | 6 | 23 | 36 | 34 | +5·9 |
| 58 | 7 | 24 | 38 | 49 | −22·4 |
| 60 | 9 | 26 | 35 | 40 | −12·5 |

Table 5. *The inorganic phosphate of normal and irradiated lenses of rabbits*

The right eye of the irradiated rabbit received 1000 r.

| Normal rabbits | Time from irradiation to death | Age at death (days) | Inorganic P (μm-moles/lens) R | Inorganic P (μm-moles/lens) L | Difference (%) $\left(\frac{R-L}{L}\times 100\right)$ |
|---|---|---|---|---|---|
| *J* | — | 14 | 303 | 295 | +2·7 |
| *K** | — | 15 | 333 | 344 | −3·2 |
| *L** | — | 15 | 349 | 363 | −3·9 |
| *M* | — | 19 | 324 | 325 | −0·3 |
| Irradiated rabbits | | | | | |
| 61* | 5 hr. | 16 | 331 | 308 | +7·5 |
| 62* | 6 hr. | 16 | 338 | 358 | −5·6 |
| 63* | 1 day | 17 | 312 | 323 | −3·4 |
| 64* | 2 days | 18 | 383 | 351 | +9·1 |

* Killed with liquid air.

Table 6. *Glutathione in normal and irradiated lenses of rabbits*

The right eye of the irradiated rabbits received 1000 r.

| Normal rabbits | Days from irradiation to death | Age at death (days) | Glutathione (μm-moles/lens) R | Glutathione (μm-moles/lens) L | Difference (%) $\left(\frac{R-L}{L}\times 100\right)$ |
|---|---|---|---|---|---|
| *A* | — | 13 | 730 | 710 | +2·8 |
| *B* | — | 14 | 772 | 755 | +2·2 |
| *C* | — | 14 | 710 | 693 | +2·5 |
| *D* | — | 15 | 837 | 856 | −2·2 |
| *E* | — | 17 | 997 | 964 | +3·4 |
| Irradiated rabbits | | | | | |
| 49 | 1 | 16 | 644 | 768 | −9·6 |
| 51* | 2 | 17 | 823 | 935 | −12·0 |
| 52 | 2 | 17 | 752 | 827 | −9·1 |
| 53 | 3 | 18 | 1000 | 1082 | −7·6 |
| 55* | 4 | 19 | 1144 | 1240 | −7·8 |
| 58 | 7 | 24 | 1637 | 1726 | −5·2 |
| 59* | 7 | 25 | 1387 | 1700 | −18·4 |

* Killed with liquid air.

*Relation between loss of GSH and loss of ~P*

In Fig. 2 the percentage difference in glutathione between the right and left lenses of normal and irradiated rabbits is plotted against the difference in ~P.

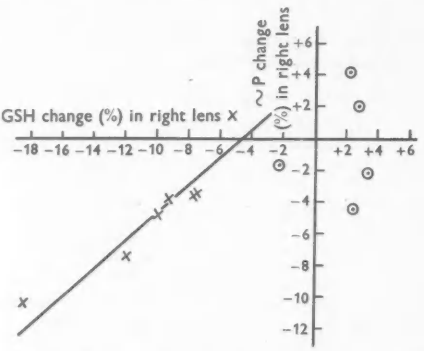


Fig. 2. Relation between percentage difference of GSH and that of ~P in the right and left lenses of normal and irradiated rabbits. ⊙, normal; ×, irradiated.

## DISCUSSION

Several points of interest arise from these results. The enzymic method makes it possible to determine the high-energy phosphate groups of ATP and ADP, the phosphate esters and glutathione in a single lens of weight about 100 mg. Unfortunately the variation between the ~P of the two lenses of a normal rabbit appears to be sufficiently large to make it impossible to detect small changes in ~P after irradiation (Table 1). There is less variation in glutathione content of the two lenses of a normal rabbit and this makes it possible to detect small changes. The results seem to show that there is a fall in glutathione 24 hr. after irradiation (Table 5). If we plot percentage difference in glutathione between normal and irradiated lenses against percentage difference in ~P (Fig. 2) the points lie on a straight line, loss in one being proportional to loss in the other; no such proportionality exists between the differences in GSH and in ~P of pairs of normal lenses. Perhaps this is a suggestion that ~P is in fact diminishing within the first few days after irradiation, although the enzymic method cannot detect this. The number of results is small but it did not seem useful to do more experiments. The point of interest is the early change and it is not possible to establish small decreases of ~P owing to the normal variation.

The results reported here, with the enzymic method of phosphate estimation, differ considerably from those obtained by the chemical fractionation described by Umbreit, Burris & Stauffer (1949), which has been so generally used. Nordmann & Mandel (1952), using Umbreit's method, have found a total of about 400 μmoles P/100 g. rabbit lens of hexose monophosphate, hexose diphosphate and phosphoglycerate, whereas the enzymic method, which we consider to be more specific, gives a value of only 25–50 μmoles P/100 g. of these phosphate esters. The enzymic method of estimation includes in this fraction phosphopyruvic and pyruvic acids, so that our values do, if anything, overestimate the amount of phosphate esters present. Kratzing & Narayanaswami (1953) noticed the same difference between the phosphate esters of brain estimated enzymically and the values reported by chemical fractionation.

Table 7. *Comparison of values obtained, by different methods of estimation, for certain phosphate compounds in tissues*

The enzymic method of Slater (1953), procedure (*a*) (see Methods), was used. The values obtained by chemical fractionation are taken from published results. Animals were killed by immersion in liquid air unless otherwise stated. Results expressed as μmoles P/100 g. wet wt.

| Tissue | Enzymic analysis (HMP, HDP, PGA, phospho-pyruvic and pyruvic acid) | Chemical analysis (HMP, HDP, PGA) |
|---|---|---|
| Skeletal muscle (rat) | 572 | — |
| | 547 | — |
| | | 517* |
| Kidney (rat) | 11 | — |
| | 16 | — |
| | 22 | — |
| | | 433* |
| Liver (rat) | 115 | — |
| | 75 | — |
| | 92 | — |
| | | 706* |
| Heart (rat) | 244 | — |
| | 135 | — |
| | 76 | — |
| | | 700* |
| Brain (guinea pig) | 26–33† | — |
| (rat) | — | 386* |
| Lens (rabbit) 25-day | 25 | — |
| 7-month | 30 | — |
| 2-year | 28 | — |
| 3-month | — | 402‡ |
| Old | — | >274‡ |

* LePage (1948).
† Kratzing & Narayanaswami (1953).
‡ Nordmann & Mandel (1952). Eyes rapidly removed and refrigerated after death of animals; 28 lenses pooled for analysis.

In an attempt to determine the significance of these differences we have estimated the phosphate-ester fraction in various tissues of the rat and compared our results with the published figures given by Umbreit's method. Table 7 shows that with skeletal muscle similar results are given by both methods, but in liver, heart and kidney there appears to be very much less of this fraction when it is estimated by the enzymic method than when it is estimated in the standard chemical way. These results show that lens is not unique in this respect. The discrepancy between chemical and enzymic determinations is a large one; in the lens, for example, about 350 $\mu$moles P/100 g., which was estimated as phosphate esters by the chemical method, now appear to be unaccounted for. We are investigating the problem further.

Creatine phosphate measured by the chemical method of Umbreit *et al.* (1949) gave the not insignificant value of 86 $\mu$moles P/100 g. of young rabbit lens (Nordmann & Mandel, 1952), although the animals were not killed in liquid air and the lenses were refrigerated until 28 lenses had been collected for a pooled analysis. The enzymic method gave very variable values, between 0 and 99 $\mu$moles CP/100 g. lens (Table 2) and precautions which usually are necessary to preserve this labile compound had no effect. It is therefore questionable whether either the chemical method of Umbreit *et al.* (1949) or the enzymic method of analysis is actually measuring creatine phosphate in the lens. The method of Ennor & Rosenberg (1952), in which the difference between free and bound creatine is measured, probably gives the most reliable value for creatine phosphate, 3–5 $\mu$moles/100 g. rabbit lens (Table 3).

## SUMMARY

1. The effect of irradiation on the organic phosphate compounds in the lens of the very young rabbit has been studied by the enzymic method of Slater (1953).

2. No change could be detected in the high-energy phosphate groups of adenosine triphosphate and adenosine diphosphate, or in hexose monophosphates, hexose diphosphate, phosphoglycerate or inorganic phosphate within a few days of irradiation.

3. A method for the enzymic determination of creatine phosphate has been worked out, but the amount in lens is too small for satisfactory determination.

4. A fall in glutathione was detected 24 hr. after irradiation.

5. The hexose phosphate and triose phosphate fraction determined enzymically is considerably less than the same fraction when determined chemically by the method of Umbreit *et al.* (1949).

6. This discrepancy between results given by enzymic and chemical methods is found in other tissues besides lens.

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# Blood Keto Acid Levels during Intoxication with Inhibitors of Carbohydrate Metabolism

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A number of compounds have come to be recognized as more or less specific inhibitors of those enzymes which depend upon SH groups for their activity. Although in many cases our knowledge of the action of these compounds is well founded, most of it is based largely on *in vitro* studies, and relatively few reports are available from which to compare the pattern of *in vivo* effects on different blood constituents produced by these various compounds in the intact animal.

Interest in this problem was aroused in this laboratory by some earlier observations on pyruvate metabolism in an unselected series of patients with peripheral neuritis (Joiner, McArdle & Thompson, 1950). In the course of this work it was found that the blood-pyruvate level was not infrequently raised in patients in whom there were no grounds for suspecting a thiamine deficiency, and in whom treatment with thiamine did not reduce the abnormal levels of pyruvate in the blood. As it is known that pyruvate metabolism can be influenced in a number of ways, apart from a deficiency of thiamine, it is possible that the lesion in such patients may be associated with the presence of some substance reacting with SH groups in an enzyme system essential for the normal metabolism of pyruvate formed from glucose.

Several reports have been made on the *in vivo* effects on the blood-sugar level of various enzyme inhibitors, including the so-called 'SH inhibitors': thus, intoxication with iodoacetate (Neuss, 1931; Irving, 1932), cyanide (Turner & Hulpien, 1933), arsenite (Oelkers, 1939), malonate (Stare & Baumann, 1940) and fluoride (Kaplan & Greenberg, 1944) all result in hyperglycaemia. The level of keto acids after intoxication with these enzyme inhibitors has not, however, been described very extensively. Peters, Sinclair & Thompson (1946) reported an increase in the level of blood pyruvate after the poisoning of pigeons, mice and rats with sodium arsenite or lewisite. Handler (1945), using the method of Friedemann & Haugen (1943), studied the changes in blood pyruvate in rabbits poisoned with cyanide, azide, fluoride, iodoacetate and malonate, while Buffa & Peters (1950) showed that poisoning with fluoroacetate had no effect on the level of keto acids in the blood of rats. With the exception of the last-mentioned workers, however, no attempt has been made to estimate pyruvic and α-oxoglutaric acids separately.

Since chromatographic methods, based on the work of Cavallini, Frontali & Toschi (1949*a*, *b*), are now available for the separation and estimation of the different keto acids in blood filtrates, it was of interest to compare the acute effects produced by the administration of a series of inhibitors of different types, including a number of SH inhibitors, on the levels of pyruvic and α-oxoglutaric acid in the blood, and also to determine any secondary changes which may take place in the blood-sugar, acetoacetate and acetone levels resulting from these interferences with carbohydrate utilization.

Some of the results obtained with the arsenite- and alloxan-poisoned animals have already been reported in a short note (El Hawary & Thompson, 1954) dealing with the nature of the substances responsible for the 'spots' in chromatograms of keto acid hydrazones extracted from blood.

## EXPERIMENTAL

*Animals.* White rats of either sex weighing 150–200 g. were used throughout.

*Sodium pyruvate* was prepared by the method of Robertson (1942) from redistilled pyruvic acid (L. Light and Co.). For injection into rats aqueous solutions containing 200 mg. sodium pyruvate/ml. were freshly prepared. The animals were injected intraperitoneally with doses of these solutions so that each received 250 mg. sodium pyruvate/kg. body wt.

### *Enzyme inhibitors*

*Arsenite.* Sodium arsenite, prepared by dissolving $As_2O_3$ in N-NaOH, was neutralized and injected subcutaneously in 0·2 ml. doses equivalent to 12 mg.$As_2O_3$/kg. body wt. The animals were killed 2 hr. later.

*Maleate.* Sodium maleate, prepared by neutralizing maleic acid (British Drug Houses Ltd.) with NaOH, was administered intraperitoneally in amounts of 0·5 ml. M maleate/100 g. body wt. (580 mg./kg.). The animals were killed after 90 min. Smaller doses of between 50 and 200 mg./kg. were without effect on the total keto acid level as determined 90–150 min. after injection.

*Malonate.* Sodium malonate (British Drug Houses Ltd.) was injected intraperitoneally in (i) doses of 1 ml. M solution/100 g. body wt. (1·04 g. malonic acid/kg.) and killed after 2 hr., (ii) two doses of 1 ml. M solution/100 g.

body wt. at 0 and 60 min., and killed after 120 min., and (iii) a dose of 1 ml. 2M malonate/100 g. and killed after 30 min.

*Iodoacetate.* Iodoacetic acid (British Drug Houses Ltd.), recrystallized from light petroleum (b.p. 100–120°) and uncontaminated with free iodine, was used. Neutral solutions of the sodium salt were prepared, and volumes varying from 0·1 to 0·2 ml. were injected intraperitoneally, so that the rats received the equivalent of 75 mg. iodoacetic acid/kg. They were killed 90 min. later.

*Alloxan.* 200 mg. alloxan (L. Light and Co.)/kg. were administered intraperitoneally in volumes varying from 0·4 to 0·5 ml. aqueous solution and the animals killed 48–72 hr. later, i.e. after hyperglycaemia had developed.

*Fluoride.* Sodium fluoride (British Drug Houses Ltd.) was given in doses (0·2–0·35 ml.) varying from 40 to 75 mg./kg. intraperitoneally, and the rats were killed after 30 min.

With the exception of the alloxan-poisoned rats the intoxication was in every case acute, and the animals were killed at times when they were showing symptoms of intoxication (lying quietly in their cages) but were not yet in a state of collapse. In no case did convulsions or any observable increase in muscular activity develop in these acutely poisoned animals. Blood required for keto acid and acetone estimations was collected directly from the cut neck into the deproteinizing solution.

### *Analytical methods*

*Blood sugar.* This was estimated by the method of Haslewood & Strookman (1939) in blood samples obtained from the tail.

*Keto acids.* These were estimated in metaphosphoric acid filtrates of blood by the method of Friedemann & Haugen (1943). Chromatographic separation and estimation of pyruvic and α-oxoglutaric acids was carried out as described by El Hawary & Thompson (1953); the second, more rapidly moving 'spot', which has since been identified as derived from pyruvic acid (LePage, 1950; Altmann, Crook & Datta, 1951; Seligson & Shapiro, 1952; Kulonen, Carpen & Ruokolainen, 1953; Markees, 1954; El Hawary & Thompson, 1954), was eluted and estimated, and its content added to that of the main pyruvate spot.

*Acetone bodies.* Acetone and acetoacetic acid were estimated in trichloroacetic acid filtrates of blood by a modification (El Hawary & Thompson, 1954) of the method of Thin & Robertson (1952).

## RESULTS

The mean values (and the standard errors of the means) for the levels of blood sugar, total keto acids (determined both chromatographically and by the method of Friedemann & Haugen), pyruvate, α-oxoglutarate and acetone bodies in normal rats and in rats poisoned with the various substances studied are shown in Table 1. In no case were any 'abnormal' spots (i.e. derived from keto acids not found in blood from normal animals) detected on the chromatograms, those derived from α-oxoglutarate, pyruvate and, in some instances, faint spots of the acetone hydrazone formed from the acetoacetate derivative being the only ones visible on the papers.

It will be seen that the levels of both pyruvate and α-oxoglutarate are significantly increased in the animals poisoned with each of the inhibitors studied. In all cases except the arsenite-poisoned animals the α-oxoglutarate level was increased to a greater extent than that of pyruvate; this was particularly so in the poisoning with maleate, which resulted in the amount of α-oxoglutarate in the blood actually exceeding that of pyruvate.

The malonate-poisoned animals referred to in Table 1 had each received the high dosage (1 ml. 2M malonate/100 g.). Doses of 1 ml. M malonate/100 g. resulted in a rise of the α-oxoglutarate level only (mean level of two poisoned animals, 1·50 mg./100 ml.), without any significant effect on the

Table 1. *Level of sugar, keto acids and acetone bodies in normal and poisoned rats*

Mean values, with standard errors of the means, are expressed in mg./100 ml. blood; keto acids are expressed in terms of pyruvic acid, and acetone bodies as acetone.

| Compound injected | No. of animals | Blood sugar | Keto acids (Friedemann & Haugen, 1943) | Total keto acids (chromatography) | Pyruvate | α-Oxoglutarate | Acetone + acetoacetate (as acetone) |
|---|---|---|---|---|---|---|---|
| None | 7 | 79±4 | 1·41±0·1 | 1·13±0·05 | 0·78±0·03 | 0·38±0·03 | 0·45±0·04 |
| Arsenite | 8 | — | 4·27±0·5 | 4·25±0·63 | 3·05±0·44 | 1·53±0·22 | 0·77±0·08 |
| Maleate | 12 | 119±2·9 | 4·24±0·17 | 4·44±0·21 | 2·08±0·12 | 3·54±0·24 | 0·80±0·06 |
| Iodoacetate | 7 | 155±4·1 | 2·73±0·02 | 2·44±0·07 | 1·55±0·08 | 1·19±0·05 | 0·69±0·03 |
| Alloxan | 8 | 420±19 | 3·43±0·23 | 2·17±0·16 | 1·33±0·17 | 1·06±0·05 | 9·51±0·69 |
| Malonate | 9 | 116±7·1 | 3·56±0·4 | 3·03±0·26 | 2·15±0·22 | 1·42±0·07 | 3·84±0·23 |
| Fluoride | 5 | 139±6·6 | 2·87±0·21 | 2·46±0·13 | 1·55±0·08 | 1·08±0·06 | 0·84±0·09 |
| Ratio of mean value in poisoned animals to mean normal value | | | | | | | |
| Arsenite | | — | 3·0 | 3·8 | 3·9 | 4·0 | 1·7 |
| Maleate | | 1·5 | 3·0 | 3·9 | 2·7 | 9·3 | 1·8 |
| Iodoacetate | | 2·0 | 1·9 | 2·2 | 2·0 | 3·1 | 1·5 |
| Alloxan | | 5·3 | 2·4 | 1·9 | 1·7 | 2·8 | 21·1 |
| Malonate | | 1·5 | 2·5 | 2·7 | 2·8 | 3·7 | 8·5 |
| Fluoride | | 1·8 | 2·0 | 2·2 | 2·0 | 2·8 | 1·9 |

pyruvate level; with the medium dosage pyruvate was slightly raised (mean level, 1·37 mg./100 ml.).

The concentration of acetone bodies in the blood was only slightly, though significantly, elevated in the animals poisoned with arsenite, maleate, iodoacetate or fluoride; in the malonate-poisoned group an eightfold increase occurred, whereas, as was to be expected from earlier work (Lackey, Bunde, Gill & Harris, 1944), in the animals poisoned with alloxan a relatively large accumulation of acetone bodies was present.

Since the blood-pyruvate level may be expected to depend on the rate of formation of pyruvate as well as on its rate of utilization it may be argued that the experiments do not necessarily demonstrate that these SH inhibitors have blocked pyruvate metabolism in the intact animal. To study the effect of these inhibitors on pyruvate utilization more precisely, blood keto acid levels were therefore also determined after raising the blood-pyruvate level by the injection of sodium pyruvate. It was found that the intraperitoneal injection into normal rats of 250 mg. sodium pyruvate/kg. body wt. resulted in a marked elevation of the blood pyruvate level 5 min. after the injection, followed by a fall so that approximately normal levels were regained after 30 min. From these resuls in normal animals, which are summarized in Fig. 1, it was decided to determine the blood-pyruvate level at 5, 15 and 30 min. after the injection of this dose of pyruvate into animals that had been poisoned with arsenite, maleate, iodoacetate and alloxan. The results of these experiments, together with the dose of inhibitor and the duration of the poisoning, are shown in Table 2. With each of these poisons it will be seen that the blood-pyruvate level remained high over the period studied. Since pure sodium pyruvate was injected in these experiments the rise in the blood keto acid level was estimated by the method of Friedemann & Haugen, although in a few experiments pyruvate was estimated after separation of the hydrazones by paper chromatography.

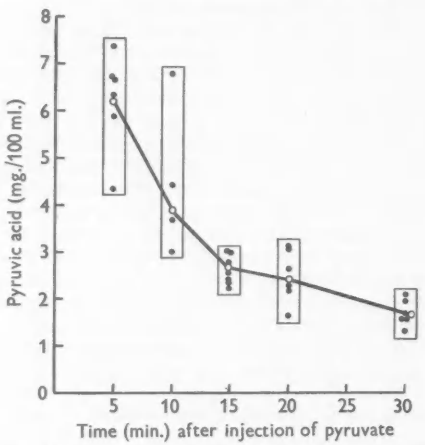


Fig. 1. Blood pyruvate levels after the intraperitoneal injection into normal rats of sodium pyruvate (250 mg./kg.); ●, level in individual rats; ○, mean level.

## DISCUSSION

As stated above, although much work has been done on the *in vitro* effects of different inhibitors of the enzymes concerned in carbohydrate metabolism, few comparisons have been made *in vivo* of the effects of these inhibitors on the levels of different intermediary metabolites in the blood. Further, as is well known, although a number of different compounds, acting by different chemical mechanisms, can inhibit various enzymes by interaction with essential SH groups in their molecules, the pharmacological and toxicological effects produced *in vivo* by these inhibitors may vary widely. Apart from possible differences in the chemical reactivity of the SH groups in different enzymes, physiological factors such as penetrability of the inhibitors to the various enzymes may well be a factor in determining these diverse toxicological reactions. It seemed, however, that a better

Table 2. *Mean blood keto acid levels in normal and poisoned rats at 5, 15 and 30 min. after the intraperitoneal injection of sodium pyruvate (250 mg./kg.)*

Expressed as mg. pyruvic acid/100 ml. blood, ±S.E.M. No. of experiments in brackets.

| Inhibitor* | Duration of poisoning (hr.) | Pyruvate levels 5 min. | 15 min. | 30 min. |
|---|---|---|---|---|
| None | — | 6·25±0·42 (6) | 2·54±0·11 (7) | 1·80±0·12 (6) |
| Arsenite | 2 | 15·76±2·13 (4) | 11·91±2·02 (5) | 9·96±1·65 (4) |
| Maleate | 1·5 | 9·26±0·85 (4) | 9·49±0·51 (4) | 11·48±1·44 (4) |
| Iodoacetate | 1·5 | 6·61±1·1 (4) | 8·75±0·71 (3) | 7·76±1·24 (3) |
| Alloxan | 2 | 8·59 (2) | 8·76±1·37 (6) | 4·78±1·19 (6) |
| Alloxan | 48 | — | 5·95±0·52 (9) | — |

* Doses of inhibitors as described in the Experimental section.

understanding of these possibilities might be reached if more were known of the changes in blood composition caused by these compounds.

Although each of the inhibitors which have been studied caused a large increase in the blood levels of both pyruvate and α-oxoglutarate, as well as a significant, though in some cases small, rise in acetoacetate, certain striking differences in the effects on these keto acid levels were noted among the various inhibitors.

The results with arsenite confirm the earlier finding of Peters *et al.* (1946) of a rapid rise in the level of pyruvate in the blood; it is here shown that the level of α-oxoglutarate also rises, and that under the conditions of poisoning used in this work the accumulation of each of these acids is about the same, i.e. to about four times the level found in normal animals. The increase in α-oxoglutarate confirms the *in vitro* effects of arsenite on α-oxoglutarate metabolism observed by Krebs & Johnson (1937), Barron & Singher (1945) and Peters & Wakelin (1952). The rise in the pyruvate level in the iodoacetate-poisoned animals is in agreement with the earlier finding of Handler (1945), and also with the *in vitro* work of Peters & Thompson (1934) and Peters, Rydin & Thompson (1935), showing that pyruvate accumulates in respiring normal brain tissue in the presence of iodoacetate.

Maleate was found by Peters & Wakelin (1946) to inhibit pyruvate oxidation by brain *in vitro*, and these results indicate that pyruvate also accumulates in the intact animal. It will be noticed, however, that with maleate the rise in α-oxoglutarate is more than three times as great as for pyruvate. Although, by analogy with arsenite, maleate might be expected to inhibit the α-oxoglutarate oxidase, it may also affect the level of α-oxoglutarate by a different mechanism. Morgan & Friedemann (1938) demonstrated that when maleic acid reacts with a monothiol, such as cysteine or glutathione, some of the acid is rearranged to give fumaric acid. Moreover, Coxon, Liébecq & Peters (1949) found that the addition of fumarate to the brain-pyruvate-oxidase system increases the amount of α-oxoglutarate in such a system metabolizing pyruvate. It seems possible, therefore, that part of the injected maleate may be converted into fumarate, the latter giving rise, through the citric acid cycle, to α-oxoglutarate, which cannot then be further metabolized owing to the inhibition of the α-oxoglutarate oxidase also produced by the maleate.

In addition to the usual changes (high blood sugar and acetone bodies) exhibited by alloxan-poisoned animals, the levels of pyruvate and α-oxoglutarate are also raised. It is not possible on present evidence to decide whether this increased pyruvate level is due to interaction of alloxan with SH groups in some component of the pyruvate-oxidase system or whether it is the outcome of the very high blood-sugar level, since it has been shown in rats (El Hawary, unpublished observations) that force-feeding with a high-carbohydate diet, or the oral administration of a large dose of glucose, can cause a comparable increase in the blood-pyruvate level. The relatively greater rise in α-oxoglutarate than in pyruvate in the alloxan-poisoned animals suggests, however, that the increased levels of these keto acids are due to a direct action of alloxan on the enzyme systems concerned.

Handler (1945) reported an increase in the blood levels of both pyruvate and acetoacetate in malonate-poisoned rabbits. The present author has confirmed these findings in rats, and has also found a rise in the α-oxoglutarate level, which probably results from the inhibition of the succinic dehydrogenase causing a diminution in the rate of α-oxoglutarate breakdown, since Busch & Potter (1952) have reported that doses of malonate, similar to those used in this work, cause a large increase in the succinate level of the tissues. It is interesting in this connexion to recall that high levels of urinary α-oxoglutarate have repeatedly been described in animals poisoned with this compound (Orten & Smith, 1937; Krebs, Salvin & Johnson, 1938; Krusius, 1940; Lee & Lifson, 1951). The relatively large accumulation of acetoacetate in malonate-poisoned animals supports Lehninger's (1946) view that pyruvic acid has two major degradative pathways in rat-liver homogenates: one through the citric acid cycle, and the other resulting in the synthesis of acetoacetate when the cycle is interrupted by malonate. The cause of this accumulation is not clear, although if there is a lack of oxaloacetate for the condensing reaction, acetylcoenzyme A units would interact to form acetoacetate (Stern, Minor & Del Campillo, 1953). If this were so, it is perhaps surprising that a comparable accumulation of acetoacetate is not found in the fluoride-poisoned animals, since it is known that fluoride inhibits both succinic dehydrogenase and α-oxoglutarate oxidase *in vitro* (Slater & Bonner, 1952; Slater & Holton, 1954). It must be remembered, however, that no estimate has been made in these experiments of the degree to which the various enzymes concerned have been inhibited by the doses of the poisons which we have used.

The experiments with injected sodium pyruvate indicate that the administration of arsenite, maleate, iodoacetate or alloxan to rats results, under the conditions used, in a gross impairment in the rate of removal of the injected pyruvate from the blood stream. It is known from the work of Johnson & Edwards (1937) that when the blood-pyruvate level is increased by exercise there is an

accompanying increase in the rate of excretion of pyruvate by the kidney, so that the return to normal of the blood-pyruvate level after the injection of sodium pyruvate will in part be brought about by increased urinary elimination. The rate at which this decline in the blood concentration occurs makes it unlikely, however, that renal excretion is playing more than a minor part in the disappearance of the injected pyruvate from the blood stream. Although impairment of renal function resulting from the action of these inhibitors may therefore in part account for the maintained high blood-pyruvate levels in the poisoned animals it seems unlikely that this is the whole explanation. These results, taken together with those given in Table 1, are therefore interpreted as providing further evidence that an *in vivo* block in pyruvate metabolism has been produced by these inhibitors.

With the exception of the alloxan-poisoned animals, the intoxication has in each case been acute and severe, so that caution is necessary in comparing the results of these experiments with the elevations of the blood-pyruvate level in human patients suffering from conditions not due to thiamine deficiency. It is possible, of course, that more prolonged exposure of rats to smaller doses of these inhibitors might also have led to raised keto acid levels, but owing to the difficulties involved in the production of such chronic intoxications in small animals, together with the danger of the production of co-existent dietary deficiencies in such long-term experiments, we have limited this phase of the work to acute intoxications.

## SUMMARY

1. A study has been made of the levels of pyruvate, α-oxoglutarate and acetoacetate in the blood of rats poisoned with different inhibitors of carbohydrate metabolism, namely arsenite, maleate iodoacetate, alloxan, malonate and fluoride.

2. Pyruvate levels have also been studied in normal and poisoned animals after intraperitoneal injection of a standard dose of sodium pyruvate.

3. Each of the inhibitors caused an increase in the blood level of pyruvate and α-oxoglutarate, the rise in the latter being particularly great in the maleate-poisoned animals.

4. Alloxan and malonate each caused a large rise in the level of acetoacetate, the other poisons studied causing only a small but significant rise in this acid.

The help and advice of Professor R. H. S. Thompson, in whose laboratory this work has been done, are gratefully acknowledged. My thanks are also due to Dr B. McArdle of the Medical Research Council Unit, Guy's Hospital, for helpful discussion of this work, and to Mr P. Hunt for his skilled technical assistance.

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